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CERTIFICATE OF MAILING

I hereby certify that on August 31, 1999, this paper (along with any paper referred to as being attached or enclosed) is being deposited with the United States Postal Service with sufficient postage in an envelope addressed to: Commissioner of Patents and Trademarks, Washington, DC 20231.

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Angela Wendel

Angela Wendel

Inventor: Curt I. Civin
Patent No.: 4,965,204
Issue Date: October 23, 1990
Patent Title: HUMAN STEM CELLS AND MONOCLONAL ANTIBODIES
Applicant: The Johns Hopkins University
Atty Docket No.: 16635-72/2

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PATENT EXTENSION
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICATION FOR EXTENSION OF PATENT TERM UNDER 35 U.S.C. § 156

Commissioner of Patents and Trademarks
Washington, D.C. 20231

Your Applicant, The John Hopkins University, represents that it is the Assignee of the entire interest in and to Letter of Patent of the United States No. 4,965,204 (the "204 Patent") granted to Curt I. Civin, on the 23rd of October 1990 for Human Stem Cells and Monoclonal Antibodies. Your Applicant, acting through the undersigned attorney, hereby proffers this Application For Extension Of Patent Term under 35 U.S.C. § 156 by submitting the following information required by 37 C.F.R. 1.740. For the convenience of the Patent and Trademark Office, the information contained in this application will be presented in a format which will follow the requirements of 37 C.F.R. § 1.740. An original of the Power of Attorney is attached hereto as "Exhibit A".

1. Identification of Approved Product. 37 C.F.R. § 1.740(a)(1).

09/15/1999 ACORBIN 00000003 162230 4965204
Sale Ref: 00000003.DOC; 162230 4965204
01 FC:111 120.00 CH

The product approved by the FDA was and is a combination product under the Safe Medical Device Amendments of 1990 (21 U.S.C. § 353 (g)(1)). As such, the product reviewed by the FDA includes the medical device (as defined at 21 U.S.C. § 321(h)) identified as the Isolex 300 and Isolex 300i Magnetic Cell Selection Systems (the "Isolex Systems") and the human biological component (as defined at 42 U.S.C. § 262(a)) identified as a suspension of human cells comprising pluripotent lympho-hematopoietic stem cells substantially free of mature lymphoid and myeloid cells, also referred to herein as a purified Peripheral Blood Progenitor Cells (PBPC) solution, which is the biological component of the cell solution generated by the Isolex Systems.

As will become evident upon consideration of Section 9 of this application, the U.S. Patent for which a term extension is being sought claims an essential component of the Isolex Systems. This essential component is the anti-CD34 mouse monoclonal antibody (anti-My-10) which is necessary for the safe and effective operation of the approved Isolex Systems. Consequently, for purposes of the present application only, the Applicant elects to designate the anti-CD34 mouse monoclonal antibody ("anti-My-10") as the Approved Product.

Reasoning and support for the Applicant's position is set forth in the Memorandum attached to this application as Exhibit B. This memorandum also provides reasoning and support for other assertions set forth in this application as set forth below.

2. Federal Statute and Applicable Provision Under Which Regulatory Review Occurred. 37 C.F.R. § 1.740(a)(2).

The IDE was submitted pursuant to Section 520(g) of the Federal Food, Drug, and Cosmetic Act (21 U.S.C. § 360j(g)). The PMA was approved under Section 515 of the Federal Food, Drug, and Cosmetic Act (21 U.S.C. § 360(e)).

3. Date Permission Received for Commercial Marketing and Use. 37 C.F.R. § 1.740(a)(3).

The Marketing Applicant[1] first received permission for commercial marketing of the Approved Product on July 2, 1999.

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[1] The Applicant, Johns Hopkins University, exclusively licensed the '204 Patent to Becton Dickinson and Company ("BD"), which, in turn, exclusively licensed the '204 Patent to Baxter Healthcare Corp. ("Baxter") in the field of use relevant to FDA approval. Baxter's Immunotherapy Division was responsible for the original FDA submission in September of 1993 and, hence, was the original Marketing Applicant. In 1997, however, Baxter's Immunotherapy Division was divested into a new entity named Nexell Therapeutics Inc. ("Nexell"). In that divestiture, Nexell acquired an exclusive sublicense from Baxter under the '204 Patent as well as Baxter's open IDE at the FDA. Hence, the current Marketing Applicant for the purposes of this application is Nexell.

4. Identification of Active Ingredient. 37 C.F.R. § 1.740(a)(4).

The active ingredient in the Approved Product is the monoclonal antibody produced by the hybridoma deposited under ATCC Accession No. HB-8483. This active ingredient has never been approved for commercial marketing or use under the Federal Food, Drug, and Cosmetic Act, the Public Health Service Act, or the Virus-Serum-Toxin Act.

5. Statement that the Application Is Being Submitted within the 60-day Period Permitted for Submission and the Last Day on which the Application Can Be Submitted. 37 C.F.R. § 1.740(a)(5).

This application for patent term extension is being submitted pursuant to 37 C.F.R. § 1.720(f) within sixty (60) days of the date July 2, 1999. The last day on which this application could be submitted is August 31, 1999.

6. Identification of Patent for which Extension Is Being Sought. 37 C.F.R. § 1.740(a)(6).

Patent Number:	4,965,204
Name of Inventor:	Curtis I. Civin
Issue Date:	October 23, 1990
Expiration Date:	October 23, 2007

7. Copy of Patent. 37 C.F.R. § 1.740(a)(7).

A copy of the patent identified in paragraph 6 hereof is attached as "Exhibit C."

8. Copies of Disclaimers, Certificates of Correction, Receipt of Maintenance Fees Payments and Re-examination Certificates. 37 C.F.R. § 1.740(a)(8).

No disclaimers have been made, nor have any certificates of correction been issued with respect to U.S. Patent 5,130,144. The maintenance fees under 37 C.F.R. § 1.20 (e) have been paid. A copy of a Certificate of Re-examination for United States Patent 4,965,204 is enclosed as Exhibit "D."

9. Statement that Patent Claims the Approved Product or Method of Using the Approved Product and Demonstration that Applicable Patent Claims Read on the Approved Product. 37 C.F.R. § 1.740(a)(9).

United States Patent Number 4,965,204 claims a monoclonal antibody that recognizes a stage-specific molecule (antigen) on immature human blood cells referred to as cluster of differentiation (CD) 34. The CD34 antigen is found on the most important of human peripheral blood progenitor cells (PBPCs) including colony forming cells for granulocytes and monocytes (CFC-GM), colony-forming cells for erythrocytes (BFU-E), colony-forming cells for eosinophils (CFC-Eo), multipotent colony-forming cells (CFC-GEMM), and other immature lymphoid precursor cells.[2] The monoclonal antibody claimed in the '204 patent is designated as the Approved Product for the purposes of this application since it is an essential component of the FDA-approved Isolex Systems. Moreover, the anti-CD34 monoclonal antibody used in the Isolex Systems, was developed in accordance with the teachings of the '204 patent and deposited with the American Type Culture Collection (ATCC) under the accession number HB-8483.

The claims of the '204 patent read directly on the Approved Product, as follows:

1. A monoclonal antibody which specifically binds to an antigen on non-malignant, immature human marrow cells, wherein said antigen is stage specific and not lineage dependent, and said antigen is also specifically bound by the antibody produced by the hybridoma deposited under ATCC Accession No. HB-8483:
   (a) which antigen is present on non-malignant, human blood or bone marrow:
      (i) colony forming cells for granulocytes and monocytes (CFC-GM),
      (ii) colony-forming cells for erythrocytes (BFU-E),
      (iii) colony-forming cells for eosinophils (CFC-Eo),
      (iv) multipotent colony-forming cells (CFC-GEMM), and
      (v) immature lymphoid precursor cells;
   (b) which antigen is present on a maximum of about 5% non-malignant, human marrow cells and a maximum of about 1% non-malignant, human peripheral blood cells; and

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[2] See A. Neil Barclay et al., The Leucocyte Antigen Fact Book. (2d ed. 1997). Academic Press.

(c) which antigen is not present on non-malignant, mature human myeloid and lymphoid cells.

2. The monoclonal antibody of claim 1 that corresponds to the monoclonal antibody produced by the hybridoma deposited under ATCC Accession No. HB-8483.

3. The monoclonal antibody of claim 1 that is the antibody produced by the hybridoma deposited under ATCC Accession No. HB-8483.

The FDA-approved labeling for the Isolex Systems describes "Anti-CD34 Monoclonal Antibody" as "an anti-human CD34 murine IgG monoclonal antibody." This monoclonal antibody binds to immature human peripheral blood cells which contain the CD34 antigen but not to non-target cells, such as mature myeloid and lymphoid cells, and is produced by the hybridoma deposited under ATCC Accession No. HB-8483. As explained in more detail in Exhibit B, the use of this monoclonal antibody is essential to the functioning of the device.

In the "Indications and Usage" section of the FDA-approved labeling, the Isolex Systems are "indicated for processing autologous peripheral blood progenitor cell (PBPC) products to obtain a CD34+ cell enriched population intended for hematopoietic reconstitution after myeloablative therapy in patients with CD34-negative tumors." The Isolex Systems achieve this result through the use of several components listed in the "Description" section of the FDA-approved labeling, including a "vial of Anti-CD34 Monoclonal Antibody." As described in the "Principles of Operation" section of the labeling, "the anti-CD34 monoclonal antibody (the primary antibody) is mixed with cells in suspension to permit binding to CD34+ cells." The CD34+ cells may then be separated from the non-target cells. Thus, claims 1-3 of the '204 patent read on the Approved Product.

10. Relevant Dates During Regulatory Review.[3] 37 C.F.R. § 1.740(a)(10).

Relevant dates and information pursuant to 35 U.S.C. § 156 (g) to enable the Secretary of Health and Human Services to determine the applicable regulatory review period are set forth below.

On October 27, 1993 an exemption was granted to initiate clinical trials of autologous stem cell concentrates for hematopoietic recovery (FDA file number BB-IDE 5272). On February 24, 1997 a Pre-market Approval (PMA) Application was filed on the Isolex 300 Magnetic Cell Separation System (FDA File Number (BP970001).

By a letter dated July 2, 1999, the FDA notified the Marketing Applicant that the PMA (BP 97-0001, BP 97-0001/01) was approved under § 515 (d) of the Federal Food, Drug and Cosmetic Act (21 U.S.C. § 360(e)). This letter is attached as "Exhibit E."

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[3] For purposes of calculating the patent term extension to which an Applicant may be entitled when seeking an extension to a patent that claims a biological product, the pertinent PTO regulations require the applicant to provide dates related to the submissions of the Investigational New Drug (IND) application, the New Drug Application (NDA) or the Product License Application (PLA) and approvals therefor. 35 U.S.C. § 156(g)(1)(B); 37 C.F.R. § 1.740(a)(10)(i). Obviously, the dates involved in the present application, however, are related to an IDE and a PMA since this is the regulatory pathway dictated by the FDA regulations for this combination product. See Exhibit B, Section III. As a result, there appears to exist a minor discrepancy in the Code as it applies to term extensions for patents that claim components of a combination product and FDA review of combination products. Obviously, this discrepancy arises simply as a result of the Code's attempt to use terminology that is consistent with the normal applicable regulatory pathway for such items. As such, it is clear that in the context of the present application, the term extension for the '204 Patent should be calculated based on the dates otherwise used for a patent directed to a medical device.

11. A Brief Description of the Significant Actives Undertaken by the Marketing Applicant During the Regulatory Review Period. 37 C.F.R. § 1.740(a)(11).

The following chronology of significant communications briefly describes the activities of the Marketing Applicant before the regulatory agency (FDA). Additional significant communications of substance that were conducted between the Marketing Applicant and the FDA relating to the approval of the Approved Product have been included as "Exhibit F."

1.	September 24, 1993:	Marketing Applicant submits original IDE application (IDE 5272)
2.	October 28, 1993:	Marketing Applicant receives verbal approval of IDE.
3.	March 25, 1994:	First patient receives selected stem cell population. Clinical studies on humans began under approved IDE protocol.
4.	February 24, 1995:	Marketing Applicant submits annual report to FDA.
5.	October 5, 1995:	Marketing Applicant sent letter to CBER requesting meeting to discuss Phase III clinical trials for IDE 5272.
6.	December 29, 1995:	Marketing Applicant submits amendment to phase-in Isolex 300i.
7.	March 12, 1996:	Marketing Applicant submits protocol change to use Isolex 300i.
8.	April 9, 1996:	Marketing Applicant submits IDE 5272 Annual Report.
9.	August 20, 1997:	Marketing Applicant submits IDE 5272 Annual Report.
10.	February 21, 1997:	Marketing Applicant files original PMA Application.
11.	March 21, 1997:	Marketing Applicant submits clinical data.
12.	June 30, 1997:	Marketing Applicant response to FDA's 6/18/97 clinical questions.
13.	July 30, 1997:	Marketing Applicant responds to FDA's 6/27/97 questions.
14.	January 16, 1998:	Marketing Applicant notified FDA of ownership change from Baxter to BIT.

15.	February 2, 1998:	Marketing Applicant submits supplement to PMA for the Isolex 300i system.
16.	March 9, 1998:	Marketing Applicant notified FDA of company name change from BIT to Nexell Therapeutics, Inc.
17.	April 13, 1998:	Day 100 meeting.
18.	June 4, 1998:	Marketing Applicant confirmation of CBER's intent for the May 22, 1998 180 day extension for review.
19.	August 27, 1998:	Response to FDA 7/2/98 letter and notification of changes to the anti-CD34 mab appearance specification.
20.	January 27, 1999:	Submission of revised package insert.
21.	June 18, 1999:	Written commitment to post-marketing studies.
22.	June 22, 1999:	Written commitment for post-marketing studies and for the GMP inspection issues.
23.	July 2, 1999:	Marketing Applicant received US FDA Pre-market Application Approval to market its Isolex 300 Magnetic Cell Separation System and the Isolex 300i Magnetic Cell Separation System.

12 (A) Statement that in the Opinion of the Applicant the Patent Is Eligible for the Extension. 37 C.F.R. § 1.740(a)(12).

Applicant is of the opinion that U.S. Patent No. 4,965,204 is eligible for extension under 35 U.S.C § 156 because it satisfies all of the requirements for such extension as follows:

(a) 35 U.S.C. § 156 (a)

U.S. Patent No. 4,965,204 claims the Approved Product. In this connection, see Exhibit B.

(b) 35 U.S.C. § 156 (a)(1)

The term of U.S. Patent 4,965,204 has not expired before submission of this application.

(c) 35 U.S.C. § 156 (a)(2)

The term of U.S. Patent No. 4,965,204 has never been extended under this provision of law.

(d) 35 U.S.C. § 156 (a)(3)

The application for extension is submitted by the owner of record in accordance with the requirement of 35 U.S.C. § 156 (d) and Rules of the U.S. Patent and Trademark Office.

(e) 35 U.S.C. § 156 (a)(4)

The Approved Product has been subject to a regulatory review period before its commercial marketing or use. In this connection, see Exhibit B, Section III.

(f) 35 U.S.C § 156 (a)(5)(A)

The commercial marketing or use of the Approved Product after the regulatory review process is the first permitted commercial marketing or use of the Approved Product under the provision of the Federal Food, Drug and Cosmetic Act (21 U.S.C. § 301) under which such regulatory review period occurred.

(g) 35 U.S.C. § 156 (c)(4)

No other patent has been extended for the same regulatory review period for the approved product.

12 (B) Length of Extension for U.S. Patent No. 4,965,204. 37 C.F.R. § 1.740(a)(12).

The length of the patent term extension of U.S. Patent 4,965,204 claimed by the Applicant is 1466 days. The length of the extension was calculated pursuant to 37 C.F.R. 1.777 as follows:

(a) The regulatory review period under 35 U.S.C. § 156 (g)(3)(B) began on October 27, 1993 and ended with the approval on July 2, 1999 which is a total of 2,074 days which is the sum of (i) and (ii) below.

(i) The period beginning on the date a clinical investigation on humans was begun (October 27, 1993) and ending on the date an application was initially submitted under § 515 (February 24, 1997). A total of 1,216 days.

(ii) The period beginning on the date an application was initially submitted under § 515 (February 24, 1997) and ending on the date such application was approved under the Act (July 2, 1999). A total of 858 days.

(b) The regulatory review period upon which the period of extension is calculated is the entire regulatory review period as determined in subparagraph 12 (B) (a), above, (2,074 days) less:

(i) The number of days in the regulatory review period which were on or before the date on which the present patent issued (December 22, 1987) which is 0 days, and

(ii) The number of days the Applicant did not act with diligence which is 0 days, and

(iii) One-half of 12 (B) (a) (i) (1,216 days) as required by 35 U.S.C. § 156 (c)(2) which is 608days;

(iv) The total of 12 (B) (a) (i) less 12 (B) (b) (i-iii) is: 2,074-0-0-608 = 1466 days.

(c) The number of days as determined in sub-paragraph 12 (B) (b) (iv) (1466 days) when added to the term of the patent (October 23, 2007), as determined by 35 U.S.C. § 154 (c)) would result in a date of October 28, 2011 which is 12 years and 113 days from the date of regulatory approval (July 2, 1999).

(d) Since the total remaining term of the patent after the addition of 1466 days to the original patent term is less than 14 years from the date of regulatory approval, and

the total extension term is less than 5 years, the expiration date of U.S. Patent No. 4,965,204 shall be October 28, 2011.

13. Applicant's Duty to Disclose. 37 C.F.R. § 1.740(a)(13).

The Applicant acknowledges a duty to disclose to the Commissioner of Patents and Trademarks and the Secretary of Health and Human Services any information which is material to the determination of entitlement to the extension sought. In this connection, the Commissioner is directed to Exhibit B.

14. Fee. 37 C.F.R. § 1.740(a)(14).

The prescribed fee for receiving and acting upon this application is to be included herein. Please charge any additional fees or underpayments, or credit any overpayment to Deposit Account 16-2230.

15. Official Correspondent. 37 C.F.R. § 1.740(a)(15).

Mr. James W. Inskeep, Esq.
Oppenheimer, Wolff and Donnelly, LLP
500 Newport Center Drive
Suite 700
Newport Beach, CA 92660-7007

(949) 823-6000
(949) 823-6031 (Direct Dial)
(949) 823-6040 (facsimile)

16. Copies of the Application. 37 C.F.R. § 1.740(a)(16).

The instant Application for Extension of the Patent Term of U.S. Patent No. 4,965,204 is being submitted as one original and duplicate copies thereof.

17. Declaration. 37 C.F.R. § 1.740(a)(17).

The requisite declaration pursuant to 37 C.F.R § 1.740 (b) is attached.

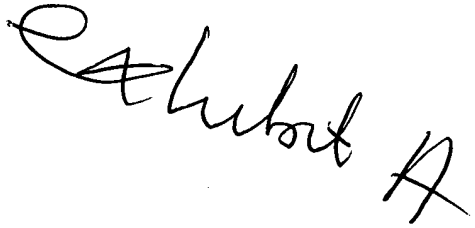
CERTIFICATION

The undersigned hereby certifies that this application for extension of patent term under 35 U.S.C. § 156 including its attachments and supporting papers is being submitted as one original and duplicate copies thereof.

8/31/99

James W. Inskeep, Esq.
Registration Number 33,910

OPPENHEIMER WOLFF & DONNELLY, LLP
2029 Century Park East, Suite 3800
Los Angeles, CA 90067-3024
Telephone: (949) 823-6000
Facsimile: (949) 823-6040



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CERTIFICATE OF MAILING

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☐ [illegible] postage as first class mail

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☒ as "Express Mail Post Office to Addressee" Mailing Label No. EL284631150US

Amanda
Angela Wendel

AUG 31 1999

PATENT

Applicant: Curt I. Civin
Patent No.: 4,965,204
Issued: October 23, 1990
Title: Human Stem Cells and Monoclonal Antibodies
Assignee: The Johns Hopkins University
Docket: 16635-72/2

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

POWER OF ATTORNEY BY ASSIGNEE AND EXCLUSION OF INVENTOR UNDER 37 C.F.R. § 1.32

Commissioner of Patents and Trademarks
Washington, D.C. 20231

Dear Sir:

The Johns Hopkins University of Baltimore, Maryland, having become the owner of all rights in and to the above-identified application by virtue of an Assignment executed by the inventor, hereby appoint the following as the attorneys of record with full power of substitution and revocation, to transact all business in the Patent and Trademark Office and before competent International Authorities connected with above-referenced patent or patent application; said appointment to be to the exclusion of the inventors and their attorneys in accordance with the provisions of 37 C.F.R. § 1.12:

W. Poms, Reg. 12,782
G. P. Smith, Reg. 20,142
H. D. Jattman, Reg. 19,777
C. A. S. Hamrick, Reg. 22,586
G. E. Lunde, Reg. 22,222
A. C. Rose, Reg. 17,047
L. J. Rovasso, Reg. 24,075
D. J. Oldenkamp, Reg. 29,421
C. Darrow, Reg. 30,166
A.C. Logan, Reg. 33,950
C.W. Chen, Reg. 41672
L. R. Hanneman, Jr., Reg. 41,063
C. Klingbeil, Reg. 33,002
C. Lerwick, Reg. 35,244
R.S. Tamura, Reg. 43,179

M. E. Brown, Reg. 28,590
H. F. O'Connor, Reg. 25,903
M. A. Kondzella, Reg. 18,013
A. P. Black, Reg. 35,450
S. R. Hansen, Reg. 38,486
D. N. Lanson, Reg. 29,401
I. W. Inskeep, Reg. 33,910
J. Boyce, Reg. 40,920
K. A. MacLean, Reg. 31,118
G.L. Fountain, Reg. 36,374
M. K. Bosworth, Reg. 28,136
J. F. Boyce, Reg. 40,920
H. Jattman, Reg. 19,777
J.P. Weir, Reg. 43,253
G.B. Wood, Reg. 28,133

R. O. Guiller, Reg. 28,852
B. C. Chang, Reg. 37,593
M. Inata, Reg. 38,190
J. D. Vorbike, Reg. 37,957
G. L. Fountain, Reg. 36,374
C. J. Lerwick, Reg. 35,244
C. Rosenberg, Reg. 31,464
M. D. Harris, Reg. 26,690
L. C. Cullman, Reg. 39,645
L.I. Peters, Reg. 33,562
C.W. Thompson, Reg. 37,802
D. Chapik is 43,424
S.M. Parker, Reg. 36233
C. Bergan, Reg. 29249

PATENT
Docket No. 16635-72/2

whose address is:

OPPENHEIMER WOLFF & DONNELLY LLP
2029 Century Park East, 38th Floor
Los Angeles, California 90067
Attn: James W. Inskeep
Telephone: (949) 719-6000
Facsimile: (949) 719-6040

The undersigned has reviewed all the documents in the chain of title of the patent application identified above and, to the best of undersigned's knowledge and belief, title is in the assignee identified above.

The undersigned, whose title is supplied below, is empowered to act on behalf of the assignee.

I hereby declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further, that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001, Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: 8/31, 1999

The Johns Hopkins University

Name: ~~Dr. Nizza Quarles~~
Title: ~~Director of the Office of Technology Licensing~~

Howard W. Cullum, Esq.
Assistant Dean and Director
Office of Technology Licensing

Exhibit B

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Exhibit B

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventor:	Curt I. Civin
Patent No.:	4,965,204
Issue Date:	October 23, 1990
Patent Title:	HUMAN STEM CELLS AND MONOCLONAL ANTIBODIES
Applicant/Assignee:	The John Hopkins University, Baltimore, Maryland

Commissioner of Patents and Trademarks
Washington, DC 20231

MEMORANDUM IN SUPPORT OF PATENT TERM EXTENSION APPLICATION

I. INTRODUCTION

In the context of the present patent term extension application for U.S. Patent No. 4,965,204 (the '204 Patent), it has become apparent to the Applicant that elaboration of assertions in the application would likely be useful to the Commissioner as it reviews the application for compliance with the statutory requirements under 35 U.S.C. Section 156. In particular, it seems that elaboration would be useful on those points related to 35 U.S.C. Section 156(a)(4) as it pertains to whether "the product has been subject to a regulatory review period before its commercial marketing or use."

In this connection, the central point is the Applicant's designation of the "Approved Product" as being the anti-CD34 mouse monoclonal antibody[1] (anti-My-10), in its role as an essential component to the safety and efficacy of the medical device component of the approved combination product, the Isolex 300 and Isolex 300i (the "Isolex Systems"). The need to make such a designation results from the fact that the product reviewed by the FDA is a combination product and thus, by definition, has at least two different components that qualify as independent "Approved Products" under the applicable FDA definitions. In this case, one of the approved products was the Isolex Systems and a second was the purified Peripheral Blood Progenitor Cell

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[1] A monoclonal antibody is an antibody derived from a single antibody producing cell (B-lymphocyte) and hence is very specific for a single antigen sub-unit, or epitope. An antigen is any substance that will induce an immune response.

(PBPC) solution that results from use of the Isolex Systems. Since the Isolex Systems were one of the components of the approved combination product, it is understood that there was corresponding approval of all essential components that are used with that device. Hence, it is asserted that one of those essential products was and is the anti-CD34 mouse monoclonal antibody. This assertion is discussed in greater detail in Section III below.

In order to provide a foundation useful for fully appreciating the content of this memorandum, the Applicant has provided a concise discussion that seeks to provide a general introduction to the pertinent therapeutic issues involved with the technology disclosed and claimed in the '204 Patent. This foundational discussion is set forth immediately below in Section II.

II. CANCER THERAPY AND THE '204 PATENT

Most normal cells of the body are in a quiescent state and only divide, if at all, when it is necessary to replace cells that have been injured, or have reached the end of their natural life cycle. Cancer results when these normally quiescent cells inexplicably start dividing in an unregulated manner. In severe forms of cancer, these unregulated cancer cells spread throughout the body via the blood and/or lymphatic systems resulting in an often-fatal form of the disease, referred to as metastatic cancer. In advanced cases of metastatic cancer, disseminated tumors can be found in virtually any organ of the body including the lungs, liver, brain and bone marrow. Therefore, treatment requires an aggressive, whole body approach using high dose chemotherapy and/or radiation. These aggressive treatments target, and effectively kill, rapidly dividing cancer cells without significant injury to the normal quiescent healthy cells that make up the majority of the body. However, any non-cancerous cells that are dividing at the time the radiation or chemotherapeutic agent is administered will be killed along with the cancerous cells.

The cells that divide and differentiate into mature blood cells are examples of normal cells that are adversely affected by high dose chemotherapy and radiation. Mature human blood cells have a relatively short life span and must be replaced frequently. Blood cell replenishment is accomplished through a process called hematopoiesis whereby blood cell precursors

(hematopoietic progenitor cells) found in the blood, bone marrow, liver and spleen constantly divide in order to replenish the mature cells of the blood. Consequently, when aggressive cancer therapy is used to treat metastatic cancer, these hematopoietic progenitor cells are killed along with the cancer significantly reducing the body's capacity to offset mature blood cell attrition.

Human blood is composed of essentially three mature cell types: red cells (erythrocytes), platelets (thrombocytes) and white cells (leukocytes). Erythrocytes carry oxygen to the various tissues of the body, thrombocytes are essential for blood clotting and leukocytes makeup the immune cells that fight disease. The leukocytes can be further divided into five mature cell types, lymphocytes (T-lymphocytes and B-lymphocytes), neutrophils, basophils, eosinophils and monocytes.

T-lymphocytes participate in the cellular immune system, which is responsible for identifying foreign materials and initiating the processes that lead to their destruction. B-lymphocytes are part of the humoral immune system and produce antibodies that protect the body from recurring infections. The neutrophil, eosinophils, basophils and monocytes engulf and destroy bacteria and other foreign invaders. Eosinophils and basophils also participate in allergic reactions and basophils are the body's primary repository of histamine.

Mature blood cells do not divide and are therefore not affected by aggressive cancer treatments. However, the hematopoietic progenitor cells that are responsible for their replenishment do divide and are therefore easily killed by chemotherapy and radiation. When this occurs, the cancer patient's mature leukocytes are rapidly diminished resulting in a compromised immune system. Consequently, many cancer patients treated with high dose chemotherapy and/or radiation develop fatal infections and die before their beleaguered immune systems can rebound from the cancer treatment.

The most successful approach to restoring a cancer patient's immune system in time to prevent fatal infections is the post treatment transplantation of healthy hematopoietic progenitor cells. This process provides the patient with fresh, viable progenitor cells that restore the immune system before infections can occur. Bone marrow is the richest sources of hematopoietic progenitor cells in the human body. However, the collection of bone marrow is a painful

procedure that few people are willing to endure and cancer patients who do donate their own bone marrow are forced to suffer yet another painful and debilitating procedure. Consequently, researchers developed a process for enriching the hematopoietic progenitor cell population in peripheral blood in order to address these negative aspects of bone marrow transplantation.[2]

In this process a donor is first administered a substance called a cytokine that stimulates the migration of progenitor cells from the bone marrow into the peripheral blood (a process called mobilization). After the donor (who is usually also the cancer patient) receives the cytokine injection his blood is monitored for the presence of hematopoietic progenitor cells using advanced laboratory techniques. When maximum hematopoietic progenitor cell levels are reached, the progenitor cell rich blood is then collected. Hematopoietic progenitor cells collected from the peripheral blood in this manner are called Peripheral Blood Progenitor Cells (PBPC's) to differentiate them from hematopoietic progenitor cells collected from bone marrow. The PBPC's are then re-administered to the patient after chemotherapy so that the patient is readily capable of reconstituting the depleted hematopoietic system.

Hematopoietic progenitor cell transplants are not without risk. Patients who receive bone marrow transplants from donor individuals (an allogenic transplant) receive the mature T-lymphocytes from the donor along with the hematopoietic progenitor cells. These foreign T-lymphocytes immediately begin attacking the transplant recipient's body resulting in graft verses host disease (GVHD), often with a fatal outcome.

Transplanting the cancer patient with native hematopoietic progenitor cells (an autologous transplant) is one technique devised to overcome GVHD. However, because autologous transplants require the collection of PBPCs from a cancer patient, there is always the risk that cancer cells may have spread into the peripheral blood. If this has occurred, unpurified, or inadequately purified, hematopoietic progenitor cell preparations may be contaminated with cancer cells that will be reintroduced into the patient potentially leading to a resurgence of cancer.

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[2] Peripheral blood is the blood that flows through the vasculature of organs and tissues and can be collected using a painless procedure analogous to donating blood.

Therefore, regardless of whether allogenic or autologous sources of hematopoietic progenitor cells are used, there are significant risks associated with these cell transplant materials unless there is a high level of certainty that the transplant materials are pure. As a result, purified solutions of allogenic transplant material depleted in mature T-lymphocytes and an autologous transplant material free of cancer cells, along with methods of formulating such solutions, are of tremendous therapeutic value. The '204 patent discloses a method for making an anti-CD34 monoclonal antibody and the use of this monoclonal in preparing a suspension of human pluripotent lympho-hematopoietic stem cells substantially free of mature lymphoid and myeloid cells (blood progenitor cells) as well as methods of achieving it. These blood progenitor cells can be distinguished from mature blood cells and many cancer cells by the presence of specific cell markers on their surfaces. The most important of these markers, and the marker used to identify the cells suspensions of the '204 patent, is designated cluster of differentiation (CD) 34 (also known as My-10).

Prior to the discoveries disclosed in the '204 patent, there were no known procedures for selectively purifying human blood progenitor cells from either bone marrow or peripheral blood in a fashion suitable for preparing substantially pure hematopoietic progenitor cell transplants. With the discovery of the CD34 antigen and the associated anti-My-10 monoclonal disclosed in the '204 patent, a new era dawned in the treatment of cancer patients. For the first time, patients with severe forms of cancer had an option to the frequently unsuccessful bone marrow transplants used in an attempt to restore their immune systems.

When the monoclonal antibody disclosed in the '204 patent[3] is used in accordance with the teachings of the '204 patent, a cell suspension greatly enriched in blood progenitor cells and depleted in non-CD34 positive cells and cancer cells results. The '204 patent discloses various methods for using the anti-My-10 monoclonal for the preparation of blood progenitor cells. For example, the anti-My-10 monoclonal can be chemically linked to a solid support such as a latex or

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[3] The monoclonal antibody disclosed in the '994 patent is designated anti-My-10 (CD34) and was derived from the HB-8483 hybridoma. A hybridoma is a cell line derived from a single B-lymphocyte that has been fused with another cell creating a stable monoclonal producing cell line. HB-8483 is an American Type Culture Collection (ATCC) assigned designation and is used to catalogue the cell line. The ATCC is a cell line repository located in Rockville, MD, U.S.A.

plastic bead. Next, bone marrow or peripheral blood containing blood progenitor cells is brought into contact with the solid support. The CD34 positive cells (blood progenitor cells) present in the blood or bone marrow bind to the solid support to which the anti-My-10 monoclonal was bound and are immobilized. The rest of the cells (CD34 negative cells) are not immobilized on the solid support and can be washed away and then discarded or reserved for other purposes.

The blood progenitor cells which were bound by the anti-My-10 monoclonal onto the surface of the solid support can be released from the solid support using a number of techniques disclosed in the '204 patent. Following their release from the solid support, the blood progenitor cells are collected in an appropriate medium such as physiological saline.[4] The suspension of substantially pure blood progenitor cells thus prepared in accordance with the teachings of the '204 patent are ready to be administered intravenously into a patient who has undergone a myeloblative procedure.[5]

The '204 patent discloses the discovery of a previously unidentified cell marker, CD34 (My-10) on the presence of human blood progenitor cells and methods for using this marker to develop an anti-My-10 monoclonal antibody. Moreover, the '204 patent discloses methods of using the anti-My-10 monoclonal thus prepared to produce a substantially pure suspension of blood progenitor cells isolated from either the bone marrow or peripheral blood of a donor. Finally, the '204 patent teaches how to use this substantially pure blood progenitor cell suspension to treat patients who have under gone aggressive cancer therapy (myeloablative procedures) which destroyed their bodies' ability to fight disease. This unique combination of teachings makes the '204 patent disclosure an invaluable advance in the treatment of the most severe forms of cancer.

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4 Physiological saline is a salt solution having a concentration of sodium chloride (table salt) at the same concentration of human body fluids.

5 Myloablative procedures include high dose chemotherapy and radiation that has killed a significant number of hematopoietic cells.

III. DESIGNATING THE ANTI-CD34 MOUSE MONOCLONAL ANTIBODY AS THE "APPROVED PRODUCT"

Having now set out the technological and therapeutic context in which the current term extension application arises, we now address the designation of the anti-CD34 mouse monoclonal antibody as the Approved Product. In that connection, this topic seems best addressed by, first, looking more closely at the use of anti-CD34 mouse monoclonal antibody in the Isolex Systems and then, the scrutiny given the antibody by the FDA. After analyzing these two areas, a clear and objective understanding can be obtained on the accuracy and appropriateness of the aforesaid designation.

A. The Anti-CD34 Mouse Monoclonal and the Isolex Systems.

As set forth in the previous section, the anti-CD34 mouse monoclonal antibody is essential to the proper implementation of the cancer treatment disclosed in the '204 Patent. More particularly, it is a critical to obtaining the purified PBPC solution disclosed in the '204 Patent for efficacious use in the reconstitution of a cancer patient's immune system. As such, so long as the Isolex System embodies a procedure disclosed in the '204 Patent for obtaining the purified PBPC solution, the importance of the anti-CD34 mouse monoclonal antibody is therefore self evident.

In this connection, the Isolex Magnetic Cell Selection Systems are "indicated for processing autologous peripheral blood progenitor cell (PBPC) products to obtain a CD34+ cell enriched population intended for hematopoeietic reconstitution after myeloablative therapy in patients with CD34-negative tumors." (See Exhibit G.) Furthermore, the Isolex System achieves this result through the use of several components listed in the "Description" section of the labeling including a "vial of Anti-CD34 Monoclonal Antibody." As discussed previously, and as described in the "Principles of Operation" section of the labeling, "the anti-CD34 monoclonal antibody (the primary antibody) is mixed with cells in suspension to permit binding to CD34+ cells." The CD34+ cells may then be separated from the non-target cells, the monoclonal released from the CD34+ cells, and the resulting solution administered to the patient. From this information, the importance of the anti-CD34 mouse monoclonal in the operation of the Isolex Systems is self evident.

B. Scrutiny Given The Anti-CD34 Mouse Monoclonal

The documentation related to the FDA's review of the Anti-CD34 mouse monoclonal is, literally, voluminous. Indeed, this monoclonal was tested under the "General Biological Products Standards" (21 C.F.R. Part 610) for safety (21 C.F.R. § 610.11), sterility (21 C.F.R. § 610.12), and purity (21 C.F.R. § 610.13). See, e.g. PMA BP970001/01 Vol. 1 at 91; PMA Vol. 43.

In this connection, the efficacy of the antibody to isolate CD34+ cells of acceptable yield, purity, and potency (in conjunction with the other components) was established in both preclinical studies (PMA Vol. 1 at 98-102; PMA Vol. 43; PMA Supp. # BP970001/01 Vol 1 at 22-24, 41-47) and clinical studies (see, e.g. PMA Vol. 1 at 103-180; PMA Vol. 9; PMA Supp. # BP970001/01 Vol 1 at 24-28, 48-59, 134-256). Furthermore, the monoclonal antibody was the subject of four volumes (Volumes 48-51) of the PMA, which described in detail the (1) method of manufacturing the antibody, (2) various biochemical tests designed to characterize the antibody, and the specifications and stability testing of the antibody. In sum, it is self-evident that FDA review and approval of the monoclonal antibody was essential to the approval of the PMA.

IV. CONCLUSION

In view of the foregoing, it is clear that the Isolex Systems embody the novel and valuable approach to obtaining and administering a purified PBPC solution to a cancer patient as set forth in the '204 Patent. Moreover, it is clear that the Isolex Systems implement this approach by relying on the use of the anti-CD34 mouse monoclonal antibody as also set forth in the '204 Patent. Finally, the evidence above shows that the FDA gave significant review to this antibody. As a result, it is entirely accurate and correct that the Approved Product for this application be designated as the anti-CD34 mouse monoclonal antibody.

Exhibit C

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United States Patent [19]
Civin

[11] **Patent Number: 4,965,204**
[45] **Date of Patent: Oct. 23, 1990**

[54] **HUMAN STEM CELLS AND MONOCLONAL ANTIBODIES**

[75] Inventor: **Curt I. Civin, Baltimore, Md.**

[73] Assignee: **The Johns Hopkins University, Baltimore, Md.**

[21] Appl. No.: **55,942**

[22] Filed: **Jun. 1, 1987**

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[52] U.S. Cl. 435/240.27; 530/387; 530/809; 424/85.8

[58] Field of Search 424/88, 89; 530/387; 435/240.27

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[57] **ABSTRACT**

Monoclonal antibodies that recognize a stage-specific antigen on immature human marrow cells are provided. These antibodies are useful in methods of isolating cell suspensions from human blood and marrow that can be employed in bone marrow transplantation. Cell suspensions containing human pluripotent lympho-hematopoietic stem cells are also provided, as well as therapeutic methods employing the cell suspensions.

6 Claims, No Drawings

HUMAN STEM CELLS AND MONOCLONAL ANTIBODIES

This application is a division of application Ser.No. 670,740, filed Feb.6, 1984, which issued on Dec. 22, 1987 as U.S. Pat. No. 4,714,680.

TECHNICAL FIELD

The present invention is directed to cell populations useful in bone marrow transplantation, as well as immortal cells producing monoclonal antibodies to human stem cells.

BACKGROUND OF THE INVENTION

Bone marrow transplantation is an effective therapy for an increasing number of diseases. Graft Versus Host Disease (GVHD), however, limits bone marrow transplantation to recipients with HLA-matched sibling donors. Even then, approximately half of the allogenic bone marrow transplantation recipients develop GVHD. Current therapy for GVHD is imperfect and the disease can be disfiguring and/or lethal. Thus, risk of GVHD restricts the use of bone marrow transplantation to patients with otherwise fatal diseases, such as malignancies, severe aplastic anemia, and congenital immunodeficiency states. Less than 1000 bone marrow transplantations per year are currently performed in the United States. Many other patients have diseases that might be treated by marrow cell transplantation (such as sickle cell anemia) if GVHD were not such a serious risk.

The potential benefits from expanded use of bone marrow transplantation have stimulated research on the cause and prevention of GVHD. It has been shown that donor T lymphocytes cause GVHD in animals. Removal of T lymphocytes from donor marrow inocula ("grafts") prevented the subsequent development of GVHD in mice, dogs and monkeys. Similar trials in humans with monoclonal antibodies against human T lymphocytes are now in progress. Preliminary results, however, suggest only attenuation of GVHD, not a cure. Similar results have been achieved with E-rosette and soybean lectin depletion of T lymphocytes. Another approach under investigation is the use of anti-T lymphocyte monoclonal antibodies conjugated to toxins, such as ricin.

As of yet, however, GVHD has not been prevented or cured in bone marrow recipients. A continuing need exists, therefore, for new methods of combatting Graft Versus Host Disease.

Donors of bone marrow are also faced with undesirable procedures and risks. The current procedures for harvesting bone marrow are expensive and painful. Furthermore, the current donation procedure is accompanied by the risks associated with anesthesia, analgesia, blood transfusion and possible infection. It would be desirable, therefore, to improve the current method of harvesting marrow from donors.

SUMMARY OF THE INVENTION

It is an object of the present invention to reduce or eliminate GVHD associated with bone marrow transplantation.

Another object of the present invention is to provide monoclonal antibodies that selectively bind immature bone marrow cells.

A further object of the present invention is to provide a method for preparing a cell population useful for stem cell transplantation that is enriched in immature marrow cells and substantially free of mature myeloid and lymphoid cells.

Yet another object of the present invention is to provide a method of collecting donations useful for stem cell transplantation that avoids the disadvantages of conventional marrow harvesting techniques.

Still another object of the present invention is to provide a therapeutic method of transplanting stem cells that can extend the use of stem cell transplantation to the treatment of non-fatal diseases.

These and other objects of the present invention are achieved by one or more of the following embodiments.

In one embodiment, the present invention provides a monoclonal antibody that recognizes an antigen on human pluripotent lymphohematopoietic stem cells, but does not recognize an antigen on normal, human mature lymphoid and myeloid cells.

The present invention also provides a monoclonal antibody to normal, immature human marrow cells that is stage-specific and not lineage dependent, said antibody (a) recognizing an antigen on normal, human blood or bone marrow (i) colony-forming cells for granulocytes/monocytes (CFC-GM), (ii) colony-forming cells for erythrocytes (BFU-E), (iii) colony-forming cells for eosinophils (CFC-Eo), (iv) multipotent colony-forming cells (CFC-GEMM), and (v) immature lymphoid precursor cells; (b) recognizing an antigen on a maximum of about 5% normal, human marrow cells and a maximum of about 1% normal, human peripheral blood cells; and (c) not recognizing an antigen on normal, mature human myeloid and lymphoid cells.

The present invention also provides a monoclonal antibody that recognizes the antigen recognized by the antibody produced by the hybridoma deposited under ATCC Accession No. HB-8483.

The present invention further provides immortal cell lines that produce the above antibodies.

In still another embodiment, the present invention provides a method of producing a population of human cells containing pluripotent lympho-hematopoietic stem cells comprising: (a) providing a cell suspension from human tissue, said tissue selected from the group consisting of marrow and blood; (b) contacting said cell suspension with a monoclonal antibody to immature human marrow cells that is stage-specific and not lineage dependent, said antibody recognizes an antigen on human pluripotent lympho-hematopoietic stem cells, but does not recognize an antigen on mature, human myeloid and lymphoid cells; and (c) separating and recovering from said cell suspension the cells bound by said antibody.

In a further embodiment, the present invention provides a method of providing a population of human cells containing pluripotent lympho-hematopoietic stem cells comprising: (a) providing a cell suspension from human tissue, said tissue selected from the group consisting of marrow and blood; (b) contacting said cell suspension with a solid-phase linked monoclonal antibody to immature human marrow cells that is stage-specific and not lineage dependent, said antibody recognizes an antigen on human pluripotent lympho-hematopoietic stem cells, but does not recognize an antigen on mature human myeloid and lymphoid cells; and (c) separating unbound cells from solid-phase linked monoclonal antibody after said contacting; and (d) recovering bound cells from

said solid-phase linked monoclonal antibody after separating said unbound cells.

Yet another embodiment of the present invention provides a suspension of human cells comprising pluripotent lympho-hematopoietic stem cells substantially free of mature lymphoid and myeloid cells, as well as therapeutic methods employing such a cell suspension.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a significant advance in the art of bone marrow transplantation. An antigen has been discovered that is expressed on immature, normal human marrow cells, including pluripotent lympho-hematopoietic stem cells (stem cells). Stem cells have the ability to restore, when transplanted, the production of hematopoietic and lymphoid cells to a patient who has lost such production due to, for example, radiation therapy. Unlike other antigens to which monoclonal antibodies have been developed, the antigen disclosed herein is not expressed by mature myeloid or lymphoid cells, yet appears on all colony-forming myeloid progenitors assayed to date. The newly discovered antigen is a stage-specific antigen that appears on bone marrow cells desirable for use in a bone marrow transplant, yet is not expressed on the more mature lymphoid cells which have been implicated as the cause of Graft Versus Host Disease. Furthermore, it has been found that the newly discovered antigen is not expressed on the peripheral blood cells that would be unnecessary or unwanted for stem cell transplantation, thus permitting the isolation of stem cells from human blood. The present invention also provides monoclonal antibodies which facilitate the isolation of the desired cells and make possible improved theraputic techniques that significantly contribute to the understanding and prevention of Graft Versus Host Disease. The isolated stem cells can also be employed to produce panels of monoclonal antibodies to stem cells.

The newly discovered antigen has been designated My-10. This antigen was identified by a monoclonal antibody raised against the KG-1*a* human leukemic cell line. The KG-1*a* cell line arose as a spontaneous tissue culture variant from the KG-1 myeloblastic leukemic cell line derived from a patient with non-lymphocytic leukemia. See Koeffler et al., (1978) *Science* 200:1153; Koeffler et al., (1980) *Blood* 56:265. Both the KG-1*a* and KG-1 leukemic cell lines are available from Dr. David Golde, at the University of California, Los Angeles.

The My-10 antigen is expressed as a cell-surface antigen on the KG-1*a* and KG-1 cell lines. The antigen is immunoprecipitated from extracts of these cell lines as a protein of approximately 115 kD (kilodalton) apparent molecular weight. The My-10 antigen is also expressed on a number of fresh acute leukemia (both lymphoid and non-lymphoid) blast cell specimens.

My-10 is expressed on very few normal human peripheral blood cells or marrow cells. Assays detect My-10 antigen on a maximum of about 5% of the normal human marrow cells and a maximum of about 1% of normal human peripheral blood cells. Various assay techniques have been employed to test for the presence of the My-10 antigen and those techniques have not detected any appreciable number (i.e., not significantly above background) of normal, mature human myeloid and lymphoid cells in My-10-positive populations. Indeed, the ability to detect My-10 antigen diminishes rapidly as blast cells differentiate into mature myeloid and lymphoid cells.

The indirect immune adherance ("panning") technique is an appropriate assay to separate the rare My-10-positive normal human bone marrow cells from the predominant My-10-negative marrow cells. Over 50% of the My-10-positive marrow cells found by this technique are blast cells of heterogeneous morphology. Only rarely are progranulocytes, promonocytes and more mature granulocytic or monocytic cells found in the My-10-positive cell fraction. Confirming results with even higher purity of isolated My-10-positive cells are achieved with immune rosetting and fluorescence-activated cell sorting (FACS).

The My-10 antigen is expressed on colony-forming cells of all marrow or blood cells lineages tested to date. For example, over 90% of the colony-forming cells-granulocyte/monocyte (CFC-GM) are isolated in the My-10-positive fraction obtained by panning marrow cells. Like CFC-GM, the colony-forming cells for pure colonies of eosinophils (CFC-Eo) are My-10-positive. Large erythroid colony-forming progenitor cells (BFU-E) are also almost uniformly My-10-positive. Mixed multipotent colony-forming cells (CFC-GEMM) also express the cell surface antigen, My-10. Only about half of the presumably more differentiated progenitors of smaller erythroid colonies ("CFU-E-like") were in the My-10-positive population obtained by panning. Erythroid cells more mature than erythroid blasts are uniformly My-10-negative. These results indicate that the cell surface My-10 expression decreases sharply between the large, immature BFU-E stage and the latter stages of erythroid maturation.

My-10 antigen is also found on immature lymphoid precursor cells. These immature lymphoid cells can be identified, for example, by detecting the presence of nuclear terminal deoxynucleotidyl transferase (TdT) as described by Bollum, (1979)*Blood* 54:1203. Approximately 5–30% of My-10-positive marrow cells have been found to be TdT-positive in several experiments. Less than 1% of the My-10-negative marrow cells were TdT-positive.

Thus, My-10 is a stage-specific antigen that is detectable on normal, human marrow or blood colony-forming cells and immature lymphoid precursor cells, but not on normal, mature human lymphoid and myeloid cells. The antigen is not lineage dependent, but appears on a spectrum of lympho-hematopoietic progenitor cells.

Anti-My-10 antibodies are extremely useful in hematopoietic research because anti-My-10 antibodies label the lympho-hematopoietic progenitor cell subset more specifically that any previously described antibody. An anti-My-10 antibody recognizes an antigen on the smallest percentage of more mature marrow cells reported and allows the isolation of relatively pure populations of immature lympho-hematopoietic cells in a single step. My-10-positive marrow cells recovered with anti-My-10 antibody can be an appropriate control normal cell population to compare with leukemic blast cells and to use in studies on the mechanisms of action of cells, factors and genes which regulate hematopoietic cell poliferation and differentiation. The near 100% recovery of most in vitro colony-forming cells in the My-10-positive marrow cell subpopulation indicates that My-10-negative accessory cells are not necessary for the growth and differentiation of these progenitor cells. Anti-My-10 antibodies also have important therapeutic

application because they allow the recovery of hematopoietic stem cell-enriched, mature lymphocyte-depleted cell population for use in human stem cell transplantation.

Anti-My-10 antibody is unique in that it recognizes an antigen on the progenitor cells CFC-GM, BFU-E, CFC-Eo, and GFC-GEMM, but does not recognize an antigen on mature, human myeloid or lymphoid cells. Anti-My-10 antibody also precipitates a protein from an extract of many human leukemic cells (e.g., KG-1 or KG-1*a* cells), and is generally found to selectively bind a maximum of about 5% normal, human marrow cells and a maximum of about 1% normal, human peripheral blood cells.

Monoclonal anti-stem cell antibodies can be produced readily by one skilled in the art. The general methodology for making monoclonal antibodies by hybridomas is now well known to the art. See, e.g., M. Schreier et al., *Hybridoma Techniques* (Cold Spring Harbor Laboratory 1980); Hammerling et al., *Monoclonal Antibodies and T-Cell Hybridomas* (Elsevier Biomedical Press 1981); Kennett et al., *Monoclonal Antibodies* (Plenum Press 1980). Immortal, antibody-secreting cell lines can also be produced by techniques other than fusion, such as direct transformation of B-lymphocytes with oncogenic DNA or EBV. Several antigen sources can be used, if desired, to challenge the normal B-lymphocyte population that is later converted to an immortal cell line.

For example, the KG-1*a* or KG-1 cell lines (preferably the KG-1*a* cell line) can be used as an immunogen to challenge the mammal (e.g., mouse, rat, hamster, etc.) used as a source for normal B-lymphocytes. The antigen-stimulated B-lymphocytes are then harvested and fused to an immortal cell line or transformed into an immortal cell line by any appropriate technique. A preferred hybridoma producing a monoclonal anti-My-10 antibody is produced by challenging a mouse with the KG-1*a* cell line and fusing the recovered B-lymphocytes with an immortal mouse plasmacytoma cell. Antibody-producing immortal cells can be screened for anti-stem cell antibody production by selecting clones that are strongly reactive with the KG-1*a* or KG-1 cells, but not reactive with granulocytes from a panel of human donors. Antibodies produced by clones which show those properties can then be screened for the additional properties of anti-stem cell antibodies.

A mouse hybridoma producing monoclonal anti-My-10 antibody was deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, on Jan. 23, 1984, and assigned ATCC Accession No. HB-8483. The present invention encompasses in a preferred embodiment any monoclonal antibody that recognizes the My-10 antigen, i.e., the antigen recognized by antibody from the hybridoma ATCC HB-8483. In another preferred embodiment, the present invention contemplates monoclonal antibodies that correspond to the monoclonal antibody produced by ATCC HB-8483; and, in a particularly preferred embodiment, the ATCC HB-8483 antibody. One antibody corresponds to another antibody if they both recognize the same or overlapping antigen binding sites as demonstrated by, for example, a binding inhibition assay.

An alternative to the above method of producing monoclonal anti-stem cell antibodies employs the My-10 antigen directly as an immunogen. The monoclonal antibody produced by hybridoma ATCC HB-8483 can be readily employed to precipitate the My-10 antigen. For example, My-10 antigen can be immunoprecipitated from cell extracts of the KG-1*a* or KG-1 cell lines, or since My-10 is expressed by many other acute leukemic cells, the antigen can be obtained from cell extracts from these sources as well. The precipitated antigen can be used as an immunogen in place of the KG-1*a* or KG-1 cell line in the above method. By application of any of the above methods, one skilled in the art can readily produce a panel of monoclonal anti-stem cell and anti-My-10 antibodies.

Another alternative is to use an anti-My-10 antibody in the production of monoclonal antibodies that recognize different antigens on stem cells and the immature marrow cells. The cells isolated from blood and marrow with anti-My-10 antibody can be used as an immunogen, as described above, to produce a panel of monoclonal antibodies against stem cells and immature marrow cells. The production of such anti-stem cell antibodies is greatly facilitated by the use of substantially pure populations of lympho-hematopoietic precursor cells provided by the anti-My-10 antibody as an immunogen. The specificities of such antibodies can be determined readily through routine screening by one skilled in the art. Thus, additional stage-specific, lineage independent antigens (and antibodies to these antigens) can be identified by those of skill in the art.

As indicated above, one application for monoclonal antibodies to stage-specific, lineage independent antigens on stem cells is the isolation of a highly enriched source of stem cells for human bone marrow transplantation. Such sources of stem cells can prevent or attenuate Graft Versus Host Disease. Anti-stem cell monoclonal antibodies (e.g., anti- My-10 antibody) can also be used to isolate stem cells for autologous reinfusion, for example, in the treatment of antigen-negative (e.g., My-10-negative) leukemias or other malignancies.

The present invention contemplates any method employing monoclonal antibodies to separate stem cells from mature lymphocytes in the marrow or blood. Generally, a cell suspension prepared from human tissue containing cells (i.e., marrow or blood cells) is brought into contact with monoclonal antibody (e.g., anti-My-10 antibody) (i) to immature marrow cells that is stage-specific, and not lineage-dependent; (ii) that recognizes an antigen or normal, human stem cells; and (iii) that does not recognize an antigen on normal, mature human myeloid and lymphoid cells. Cells which have been bound by the monoclonal antibody are then separated from unbound cells by any means known to those skilled in the art.

Various methods of separating antibody-bound cells from unbound cells are known. For example, the antibody bound to the cell (or an anti-isotype antibody) can be labeled and then the cells separated by a mechanical cell sorter that detects the presence of the label. Fluorescence-activated cell sorters are well known in the art. In one preferred embodiment, the anti-stem cell antibody is attached to a solid support. Various solid supports are known to those of skill in the art, including, but not limited to agarose beads, polystyrene beads, hollow fiber membranes and plastic petri dishes. Cells that are bound by the antibody can be removed from the cell suspension by simply physically separating the solid support from the cell suspension. Preferred protocols, however, will be described.

Selective cytapheresis can be used to produce a cell suspension from human bone marrow or blood contain-

ing pluripotent lymphohematopoeitic stem cells. For example, marrow can be harvested from a donor (the patient in the case of an autologous transplant; a donor in the case of an allogenic transplant) by any appropriate means. The marrow can be processed as desired, depending mainly upon the use intended for the recovered cells. The suspension of marrow cells is allowed to physically contact, for example, a solid phase-linked monoclonal antibody that recognizes an antigen on the desired cells. The solid phase-linking can comprise, for instance, adsorbing the antibodies to a plastic, nitrocellulose or other surface. The antibodies can also be adsorbed on to the walls of the large pores (sufficietly large to permit flow-through of cells) of a hollow fiber membrane. Alternatively, the antibodies can be covalently linked to a surface or bead, such as Pharmacia Sepharose 6MB macrobeads ®. The exact conditions and duration of incubation for the solid phase-linked antibodies with the marrow cell suspension will depend upon several factors specific to the system employed. The selection of appropriate conditions, however, is well within the skill of the art.

The unbound cells are then eluted or washed away with physiologic buffer after allowing sufficient time for the stem cells to be bound. The unbound marrow cells can be recovered and used for other purposes or discarded after appropriate testing has been done to ensure that the desired separation had been achieved. The bound cells are then separated from the solid phase by any appropriate method, depending mainly upon the nature of the solid phase and the antibody. For example, bound cells can be eluted from a plastic petri dish by vigorous agitation. Alternatively, bound cells can be eluted by enzymatically "nicking" or digesting a enzyme-sensitive "spacer" sequence between the solid phase and the antibody. Spacers bound to agarose beads are commercially available from, for example, Pharmacia.

The eluted, enriched fraction of cells may then be washed with a buffer by centrifugation and either cryopreserved in a viable state for later use according to conventional technology or immediately infused intravenously into the transplant recipient.

In a particularly preferred embodiment, stem cells can be recovered directly from blood using essentially the above methodology. For example, blood can be withdrawn directly from the circulatory system of a donor and percolated continuously through a device (e.g., a column) containing the solid phase-linked monoclonal antibody to stem cells and the stem cell-depleted blood can be returned immediately to the donor's circulatory system using, for example, a conventional hemapheresis machine. When a sufficient volume of blood has been processed to allow the desired number of stem cells to bind to the column, the patient is disconnected. Such a method is extremely desirable because it allows rare peripheral blood stem cells to be harvested from a very large volume of blood, sparing the donor the expense and pain of harvesting bone marrow and the associated risks of anesthesia, analgesia, blood transfusion, and infection. The duration of aplasia for the transplant recipient following the marrow transplant can also be shortened since, theoretically, unlimited numbers of blood stem cells could be collected without significant risk to the donor.

The above methods of treating marrow or blood cell suspensions produce a suspension of human cells that contains pluripotent lympho-hematopoietic stem cells, but substantially free of mature lymphoid and myeloid cells. The cell suspension also contains substantially only cells that express the My-10 antigen and can restore the production of lymphoid and hematopoietic cells to a human patient that has lost the ability to produce such cells because of, for example, radiation treatment. By definition, a cell population that can restore the production of hematopoietic and lymphoid cells contains pluripotent lympho-hematopoietic stem cells.

The above cell populations containing human pluripotent lympho-hematopoetic stem cells can be used in therapeutic methods such as stem cell transplantation as well as others that are readily apparent to those of skill in the art. For example, such cell populations can be administered directly by I.V. to a patient requiring a bone marrow transplant in an amount sufficient to reconstitute the patient's hematopoietic and immune system. Precise, effective quantities can be readily determined by those skilled in the art and will depend, of course, upon the exact condition being treated by the therapy. In many applications, however, an amount containing approximately the same number of stem cells found in one-half to one liter of aspirated marrow should be adequate.

The following examples are provided to illustrate specific embodiments of the present invention. The examples are included for illustrative purposes only and are not intended to limit the scope of the present invention.

EXAMPLE I

Development of an Anti-My-10 Monoclonal Antibody

The monoclonal antibody, anti-My-10, was produced by hybridizing SP-2 plasmacytoma cells with splenocytes from a BALB/cJ mouse which had been repeatedly immunized with viable KG-1*a* cells. Four to twelve week old BALB/cJ female mice were obtained from the Jackson Laboratories (Bar Harbor, Maine), and utilized for development and production of monoclonal antibodies. KG-1*a* cells were obtained from Dr. D. Golde (UCLA).

Antibody secreting hybridomas were produced by fusion of mouse plasmacytoma cells with splenocytes, using the techniques of Kohler and Milstein, (1975) *Nature* 256:495, as modified by Fazekas de St. Groth and Scheidegger, (1980) *J. Immunol. Methods* 35:1. A BALB/cJ female mouse was hyperimmunized by intraperitoneal injections (four injections over a four month period) of approximately 10 million washed, viable KG-1*a* cells in saline; the fourth of these injections was five days prior to fusion. Three and four days prior to fusion, the mouse was boosted intravenously with KG-1a cells. Then, the mouse spleen cells were fused with non-immunoglobulin-producing SP-2/0-AG14 (SP-2) mouse plasmacytoma cells and cultured in HAT medium. Fazekas de St. Groth and Scheidegger, (1980) *J. Immunol. Methods* 35:1. Hybridomas were assayed, and the anti-My-10-producing clone was selected for binding to KG-1a cells, but not to human peripheral blood granulocytes. The hybridoma cells were subcloned at least twice. Neat spent hybridoma culture supernate was used as the source of antibody, under conditions (determined in preliminary experiments) sufficient to saturate binding sites on KG-1a cells. The isotypes of all hybridoma-and plasmacytoma-derived antibodies used were determined as previously de-

scribed. Civin and Banquerigo, (1983) *J. Immunol. Methods* 61:1.

By two weeks, macroscopic colonies were observed in all 48 cultures; the culture supernates were tested in indirect immunofluorescence assays on KG-1a cells, as well as on granulocytes from several normal donors. Four of these initial culture supernates were strongly reactive (at least five times background) with KG-1a cells, but did not react with granulocytes from any donor tested. The hybridoma culture producing the anti-My-10 monoclonal antibody was cloned in soft agarose. Civin and Banquerigo, (1983) *J. Immunol. Methods* 61:1. Anti-My-10 was shown to be an IgG 1 (Kappa) antibody, by enzyme-linked immunosorbent assay, Civin and Bangerigo, (1983) *J. Immunol. Methods* 61:1, using isotype-specific antibodies (Zymed Laboratories, Burlingame, Calif.). The thrice-cloned hybridoma producing monoclonal anti-My-10 antibody is available from the American Type Culture Collection under ATCC Accession No. HB-8483.

EXAMPLE II

Expression of My-10 Antigen on Myeloid Cell Lines and Normal Human Blood and Marrow Cells

Cell lines were obtained and cultured as previously described. Strauss et al., (1983) *Blood* 61:1222. In addition, the recently described HEL human erythroleukemia cell line (Martin and Papayannopoulou, (1982) *Science* 212:1233) was generously provided by Dr. T. Papayannopoulou (Seattle, Wash.), and was cultivated similarly.

Heparinized (20 units/ml) peripheral blood was obtained from normal laboratory volunteers, and cell types were separated by several techniques. Platelets, red blood cells and peripheral blood mononuclear cells (PBMC) were separated as described previously (Civin et al., (1981) *Blood* 57:842; Strauss et al., (1983) *Blood* 61:1222) over Histopaque-1077 ® (Sigma, St. Louis, Mo.). Since Todd et al., (1981) *J. Immunol.* 126:1435, had pointed out that monocytes may adsorb platelet fragments during conventional PBMC preparation as above, defibrinated (rather than heparinized) blood samples were used when monocytes were to be evaluated. Lymphocytes or monocytes in a mixed population of PBMC could be separately analyzed for flourescence by first selecting a "lymphocyte region" or "monocyte region," on the basis of forward and right angle light scatter (Hoffman and Hansen, (1981) *Int. J. Immunopharmac* 3:249) using flow cytometry (Spectrum III cytofluorograph; Ortho Diagnostics, Raritan, N.J.). In other studies, the monocytes/macrophages in PBMC preparations (1million cells/ml complete growth medium) were labelled by incubation (37° C., 5% CO_2, 45 min.) with 100 million/ml latex microspheres (Dow Diagnostics, Indianapolis, Ind.). After washing, phagocytic mononuclear cells were identified microscopically (at least 3 beads/cell).

To obtain enriched T- and B-lymphocyte populations, PBMC (5 million/ml complete growth medium) were first depleted of monocytes and macrophages by incubation (37° C., 5% CO_2, 90 min.) in plastic petri dishes (Falcon, Oxnard, Calif.). The nonadherent PBMC were then washed and fractionated using sheep erythrocyte (E)-rosette formation. Jondal et al., (1972) *J. Exp. Med.* 136:207. To isolate peripheral blood granulocytes, mononuclear cells were first removed by Histopaque-1077 200 density gradient centrifugation. The cells beneath the interface of the first gradient were washed once, and granulocytes were then separated from red cells by dextran sedimentation. Small numbers of residual red cells did not interfere with later analysis of antibody binding to leukocytes; if large numbers (greater than 25%) of red cells were present, they were lysed osmotically. Crowley et al., (1980) *New Eng. J. Med.* 302:1163.

Marrow was aspirated from posterior iliac crests into alpha medium (M. A. Bioproducts, Walkersville, Md.) containing preservative-free heparin (100 units/ml Panheprin ®; Abbott, Chicago Ill.). Excess cells obtained from donor marrow harvested for allogeneic marrow transplantation, or marrow cells from normal volunteers were utilized. Diluted marrow samples were centrifuged over Histopaque-1077 ®. The interface cells were washed, suspended in complete growth medium, and incubated (37° C., 5% CO_2) in petri dishes for at least 90 min. to remove plastic-adherent cells. The low density, plastic nonadherent marrow cells were washed at least once again prior to use. Leukemic blast cells were obtained from patient diagnostic marrow samples as previously described. Civin et al., (1981) *Blood* 57:842.

The antibodies I2 (Nadler et al., (1981) *Prog. Hematol.* XII: 187-225, anti-HLA-DR), cALLa (Ritz et al., (1980) *Nature* 283:583, anti-common acute lymphoblastic leukemia antigen), Mo2 (Todd et al., (1981) *J. Immunol.* 126:1435, monocyte-specific), T11 (Kamoun et al., (1981) *J. Exp. Med.* 153:207; Howard et al., (1981) *J. Immunol.* 126: 2117, anti-sheep red blood cell receptor of T-cells), and B1 (Nadler et al., (1981) *Prog. Hematol.* XII: 182-225, anti-pan B-cell) were generously provided by Dr. L. Nadler (Sidney Farber Cancer Center, Boston, Mass.) and Dr. K. Kortwright (Coulter Diagnostics, Hialeah, Fla.). The anti-leu-1 monoclonal antibody (Engleman et al., (1981) *Proc. Natl. Acad. Jei. USA* 78:1891) was generously provided by Dr. R. Levy (Stanford, Palo Alto, Calif.). The MOPC 21 IgG 1 (Kappa) mouse myeloma protein, produced by P3×63.AG8 cell line (American Type Tissue Collection, Rockville, Md.) and having no known specificity, was utilized as a negative control antibody (culture supernate). The 28/43/6 monoclonal antibody, which binds to lymphocytes from all donors tested (Strauss et al., (1983) *Blood* 61:1222), was used as a positive control.

Indirect immunofluorescence assays to measure binding of monoclonal antibodies to cells were performed as previously described. Civin et al., (1981) *Blood* 57:842; Strauss et al., (1983) *Blood* 61: 1222. Binding was analyzed either by standard phase and fluorescence microscopy and/or by flow microfluorometry.

Large quantities of cell surface My-10 antigen (indirect immunofluorescence assay) were detected by flow microfluorometry and other methods on KG-1a cells. The anti-My-10-labelled KG-1a cell population was even (slightly) more intensely fluorescent than the (positive control) 28/43/6-labelled sample (Table 1). In contrast, when the other cell lines were labelled with anti-My-10, neither the fluorescence histograms nor the derived values were greatly different from the negative control (MOPC 21) profile. (Daudi and K-562 cells were not detectably labelled with the positive control 28/43/6 antibody. This is consistent with the thesis that this antibody detects a framework epitope of the HLA-A,B molecule, since HL-A-A,B is not expressed on Daudi or K0562 cells. Strauss et al., (1983) *Blood* 61:1222). In this experiment, Daudi cells appeared

slightly positive for MY-10. However, in other experiments, all of these cell lines (except KG-1a) were clearly negative for anti-My-10-binding. The same conclusions were reached when whole viable cells were tested by enzyme-linked immunoassays (EIA), and treated marrow cells were more fluorescent than the 99.9 percentile cell treated with MOPC 21. FACS II oscilloscope fluorescence vs. light scatter "dot plots" of these marrow cells at two FACS II laser voltage settings were made.

TABLE 1

My-10 Antigen Expression on Human Leukemic Cell Lines: Derived Population Fluorescence*

	MOPC 21 (Negative Control)		Anti-My-10		28/43/6 (Positive Control)	
	Mean Fluorescence Intensity**	Percent Bright Cells***	Mean Fluorescence Intensity	Percent Bright Cells	Mean Fluorescence Intensity	Percent Bright Cells
KG-1a	1.2	[10%]	10.0	92%	9.5	81%
U-937	1.5	[10]	1.8	17	17.1	97
Daudi	1.0	[10]	1.8	22	1.1	13
ML-1	0.8	[10]	1.0	15	4.0	91
MOLT-3	1.0	[10]	1.9	19	5.0	77
HEL	1.9	[10]	3.3	21	12.2	78
HL-60	2.9	[10]	1.6	2	29.0	86
K-562	3.3	[10]	2.3	9	1.9	2

*Values derived from histograms.
**Normalized mean population intensity of fluorescence. See Durand, (1982) Cytometry 2: 192.
***Percent of cells brighter than 90th percentile fluorescence with negative control (MOPC21) monoclonal antibody.

TABLE 2

My-10 Antigen Expression on Blood and KG-1 Cells: Derived Population Fluorescence*

	MOPC 21		Anti-MY-10		28/43/6	
	Mean Fluorescence Intensity	Percent Bright Cells	Mean Fluorescence Intensity	Percent Bright Cells	Mean Fluorescence Intensity	Percent Bright Cells
Lymphocytes	0.3	[10%]	0.3	8%	NDA**	ND
Granulocytes	1.0	[10]	0.9	6	ND	ND
Monocytes	1.4	[10]	1.4	13	17.5	86%
KG-1	0.8	[10]	2.0	13	5.4	87

*Values derived from histograms and calculated as in Table 1.
**Not done.

when purified anti-My-10 was used rather than tissue culture supernate.

Table 2 shows FACS fluorescence data of isolated peripheral blood granulocytes, plastic-adherent monocytes (86% monocytes by Wright-Giemsa stain), and nonadherent "lymphocytes" (66% lymphocytes by Wright-Giemsa stain) after reaction with anti-My-10. No specific fluorescence was detected. In several additional immunofluorescence and EIA assays, anti-My-10 did not label peripheral blood granulocytes, mononuclear cells (including E-rosette-positive and E-rosette-negative cells, and latex bead-labelled phagocytic cells, analyzed individually), red cells, or platelets from any of 9 normal human blood donors.

Low-density, plastic-nonadherent, marrow cells from normal human donors were analyzed for cell surface expression of My-10 antigen by indirect immunofluorescence using visual microscopic detection. A small, but definite (1.3% mean) subpopulation of marrow cell was fluorescent over MOPC 21 background in eight experiments. A small subpopulation of My-10-positive marrow leukocytes was also identified by flow cytometry. KG-1a cells, tested in the same experiment, are shown for comparison. In both the KG-1a cells and the My-10-positive marrow cells, there is cellular heterogeneity with regard to My-10 antigen cell surface density, from near background to off-scale at these instrument settings. Mean fluorescence intensity of the anti-My-10-treated marrow cells was 1.2, compared to 0.8 with MOPC 21 and 15.6 with 28/43/6. 2.1% of anti-My-10-

EXAMPLE III

Morphologic and Cytochemical Phenotype of My-10-Panned Marrow Cells

The technique of Engleman et al., *Proc. Natl. Acad. Sci. USA* 78:1891, was utilized as previously described. Strauss et al., (1983) *Blood* 61:1222. Briefly, to non-tissue culture-treated plastic petri dishes (Lab-Tek, Naperville, IL.; 60 mm) was added 5 ml of sterile Tris buffer (0.1M, pH 9.2) containing 20 ug/ml affinity-purified goat anti-mouse IgG antibody (Kirkegaard & Perry). After 45 minutes (22° C.), the dishes were rinsed three times with Hank's balanced salt solution (HBSS), then once with HBSS containing 0.2% Bovine serum albumin (BSA), and stored (4° C.) in the latter medium. Immediately prior to use, dishes were washed with HBSS containing 0.2% BSA.

Plastic-nonadherent, low density marrow leukocytes were adjusted to 5 million/ml in HBSS containing 0.2% BSA and incubated (30 min., 22° C.) with an equal volume of spent hybridoma supernate (conditions of antibody excess, as determined in preliminary experiments). Cells were then washed twice in cold HBSS containing 0.2% BSA. Ten million cells in 2 ml of the same cold medium were placed in a goatanti-mouse Ig-coated petri dish at 4° C. The dish was rocked gently after one hour, and after two hours, the unbound cells were harvested by rocking and gentle pipetting with

three 2 ml volumes. The bound cells were released by 3 rinses with vigorous pipetting.

Only 1.7–2.2% of the normal human low density, plastic nonadherent, bone marrow cells bound to the My-10 panning plates in these four experiments. Cell fractions were then cytocentrifuged and stained for morphology (Table 3). The small My-10-positive marrow cell fraction contained many undifferentiated blast cells (Table 3). Small numbers of progranulocytes, more mature granulocytic cells, and lymphoid cells were also observed in this cell fraction. These results were confirmed by analysis of double esterase cytochemical stains of the cell fractions (Table 4) which suggested the presence of both monoblasts (nonspecific esterase-positive) and myeloblasts (NASD chloroacetate esterase-positive).

Smeared or cytocentrifuged preparations of whole or separated marrow cells or colonies were stained either with Wright-Giemsa stain, or with a double-esterase (alpha-naphthyl acetate and naphthol AS-D chloroacetate esterases) cytochemical stain and Mayer's Hematoxylin counterstain for differential counting, or with other cytochemical stains (Yam et al., (1971) *Am. J. Clin. Path.* 55:283).

TABLE 3

Differential Blood Counts* of My-10-Antigen-Positive vs. Negative Marrow Cells

	Unseparated		My-10-Neg.		My-10-Pos.	
Marrow Cells:	Exp 1***	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2
Blast cells	2	2	7	6	74	62
Promyelocytes	4	4	7	3	5	7
Myelo-Neutro**	53	54	40	44	6	2
Basophils/Eosinophils	1	0	1	1	1	1
Monocytes	9	3	4	1	4	0
Lymphocytes	25	27	34	43	7	26
Plasmacytes	1	1	0	0	1	1
Erythroblasts	3	11	6	3	1	0

	Unseparated		My-10-Neg.		My-10-Pos.	
Marrow Cells:	Exp 3	Exp 4	Exp 3	Exp 4	Exp 3	Exp 4
Blast cells	18	10	1	7	23	65
Promyelocytes	6	24	10	15	16	14
Myelocytes	4	17	11	4	4	0
Metamyelocytes	23	2	5	2	12	0
Band forms	1	0	12	0	16	0
Segmented neutrophils	36	1	0	0	3	0
Basophils	0	2	1	0	2	0
Eosinophils	0	0	3	0	2	0
Monocytes	0	3	0	2	1	2
Lymphocytes	3	25	29	42	22	16
Plasmacytes	0	1	0	1	0	1
Erythroblasts:						
Orthochromatophilic	7	12	26	20	0	0
Basophilic	1	3	2	6	0	0
Polychromatophilic	2	0	0	1	0	1
Proerythroblasts	0	2	1	0	0	0

*Percent of at least 200 Wright's-stained cells counted (rounded).
**Myelocytes, metamyelocytes, band forms, plus segmented neutrophils.
***Exp = Experiment.

TABLE 4

Cytoplasmic Esterase Content of My-10-Antigen-Positive vs. Negative Marrow Cells*

	Unseparated		My-10-Neg.		My-10-Pos.	
Marrow Cells:	Exp 3	Exp 4	Exp 3	Exp 4	Exp 3	Exp 4
Naphthol AS-D Chloroacetate esterase-positive**	66	34	40	16	36	2
Alpha naphthyl acetate esterase-positive						

TABLE 4-continued

Cytoplasmic Esterase Content of My-10-Antigen-Positive vs. Negative Marrow Cells*

	Unseparated		My-10-Neg.		My-10-Pos.	
Marrow Cells:	Exp 3	Exp 4	Exp 3	Exp 4	Exp 3	Exp 4
Diffusely Stained	1	6	1	0	5	0
Focally Stained	0	0	7	1	2	0
Unstained	33	62	52	83	55	98

*Percent of 200 cells counted (rounded). Data for experiments 3 and 4 are from the experiments designated by the same numbers in Table 3.
**"Nonspecific esterase."

EXAMPLE IV

Anti-My-10-Immune Rosetting Human Marrow Cells

Previously described procedures (Goding, (1976) *J. Immunol. Methods* 10:61; Parish and McKenzie, (1978) *J. Immunol. Methods* 20: 173) were modified as described below. Human O-negative red cells were purified from heparinized fresh whole blood by centrifugation (300 ×g 30 min., 22° C.) over Mono-Poly-Resolving Medium (Flow Laboratories, McLean, VA). The leukocyte-free, erythroid cell pellet was washed five times in sterile 0.9% NaCl (4° C., 300×g, 10 min.) and stored 16 hours as a 10% suspension in isotonic saline (4° C.). Affinity-purified goat anti-mouns IgG (Kirkegaard and Perry), and protein A-sepharose column (Pharmacia, Piscataway, NJ) -purified (Ey et al., (1978) *Immunochem* 15:429) monoclonal antibody (anti-My-10, MOPC 21, or 28/43/6) in isotonic saline were centrifuged (15,600×g, 30 min., 4° C.) to remove macroaggregates immediately prior to use. Immune red cells were prepared by the dropwise addition of 0.5 ml 0.01% chromic chloride to a (4° C.) suspension containing 350 ul isotonic saline, 50 ul freshly washed packed red cells, and 50 ul antibody (1 mg/ml). After five min. (22° C.), an equal volume of phosphate-buffered saline (PBS) containing 0.1% sodium azide was added to stop the reaction. The immune red cells were washed by centrifugation, transferred to a fresh test tube, then washed again and resuspended to a 10% suspension in PBS containing 0.1% sodium azide and 10% fetal bovine serum (FBS). All manipulations were under aseptic conditions. The immune red cells were kept at 4° C. until use later that day.

In the direct immune rosetting procedure, one million low density, plastic-nonadherent marrow cells in 100 ul PBS containing 0.1% sodium azide and 10% FBS were mixed with 50 ul immune red cell suspension. After gentle centrifugation (200×g, 5 min., 4° C.), cells were mixed gently, then kept at 4° C. for one hour. Next, 3 ml of HBSS containing 0.2% bovine serum albumin was added. Aliquots were cytocentrifuged and stained for morphological analysis. To the residual volume, one drop of 1% gentian violet was added, and wet mounts were prepared and counted.

For the indirect immune rosetting procedure, cells were first incubated with centrifuged McAb (60 min., 4° C.), washed twice, then rosetted with goat-anti-mouse IgG-coated red cells as in the procedure for direct rosettes.

1.5–3% of nucleated marrow cells were My-10-positive by these assays. Morphologic analysis of cytocentrifuged rosette preparations indicated that few mature cells formed rosettes and that the predominant My-10-positive cells were blast cells (Table 5), although not all

blast cells were My-10-positive (by either panning or immune rosetting).

TABLE 5

Anti-My-10 Immune Rosetting-Human Marrow Cells: Differential Nucleated Cell Counts*

	Anti-My-10-Rosetting Marrow Cells**		
	Direct Assay	Indirect Assay	Whole Marrow
Blast Cells	48%	63%	10%
Promyelocytes	11	8	10
Myelocytes	0	0	13
Metamyelocytes	0	1	16
Band forms and segmented neutrophils	11	8	18
Monocytes	0	0	15
Lymphocytes	8	1	10
Orthochromatophilic normoblasts	2	2	6
Polychromatophilic normoblasts	0	0	3
Unidentifiable***	20	17	0

*Based upon 200 cell counts (rounded). Whole marrow was taken as the first 200 nucleated cells seen on the indirect anti-My-10 test slide, whether rosetted or not.
**In wet mounts from this experiment, 1.5% of marrow cells formed direct anti-My-10 rosettes, and 3.0% formed indirect anti-My-10 rosettes. Comparison results using MOPC 21 control rosettes were 0% (direct and indirect), and using 28/43/6 rosettes were 100% (direct and indirect).
***Morphology obscured by rosetted erythrocytes.

EXAMPLE V

Expression of My-10 by Human Myeloid Colony-Forming Cells for Granulocytes and Monocytes (CFC-GM)

Normal marrow cell fractions obtained as above were assayed for CFC-GM in semisolid agar cultures.

Day 12-14 CFC-GM were assayed in triplicate in semi-solid agar with 5% placenta-conditioned medium (Pike and Robinson, (1970) *J. Cell. Physiol.* 76:77; Burgess et al., (1977) *Blood* 49:573) exactly as described previously (Strauss et al., (1983) *Blood* 61: 1222). Day 14 multilineage colonies (Fauser and Messner, (1979) *Blood* 53:1023; Nakahata et al., (1982) *Blood* 59:857; Iscove, et al., (1974) *J. Cell. Physiol.* 83:309) were assayed in quadruplicate in medium containing 0.96% methylcellulose, 5% placenta-conditioned medium, and 1 unit/ml erythropoietin (Connaught, Torronto, ONT). Colony number was a linear function of total cells plated. It should be noted that, in most experiments, cells were plated at several dilutions to obtain countable plates (20-200 colonies). This was particularly important with My-10-positive cell fractions, which were enriched in colony-forming cells. In addition, mixed lineage colonies were not scored on plates with more than 100 total colonies per plate, to avoid scoring superimposed colonies as products of a single colony-forming cell.

Colonies were counted in situ using a dissecting microscope (50-80×) or inverted phase microscope (200×), and gross colony and cellular morphology was recorded. Representative colonies were plucked using a Pasteur pipette. Stained cytocentrifuge preparations were analyzed for confirmation of cell type(s) within the colonies.

Less than 10% of the CFC-GM were detected in the My-10-negative cell fraction, and the My-10-positive cell fraction was several-fold enriched for CFC-GM, compared to unfractionated marrow or control IgG1 (MOPC 21)-bound marrow cells (Table 6). However, only approximately 40% of the CFC-GM of the initial marrow sample were recovered in the My-10-positive cell population. This might be explained by mechanical injury to the My-10-positive cells or by partition of an accessory cell type Sharkis et al., (1981) In Gershwin and Merchant (eds), *Immunologic Defects in Laboratory Animals* (Plenum, NY) 1:79; Strauss et al., (1983) *Blood*, in press).

Marrow cell fractions obtained by My-10-panning were also cultured in medium containing methylcellulose. As in agar cultures, CFC-GM were almost totally depleted from the My-10-negative fraction (Tables 7,8). In the experiment shown in Table 7, the My-10-positive fraction was approximately 30-fold enriched in CFC-GM and contained 90% of the initial CFC-GM (the full recovery of CFC-GM in this experiment contrasted with yields of CFC-GM in agar cultures described above). CFC-GM colony subtypes (granulocyte, monocyte vs. granulocyte/monocytes [data not shown]; small vs. large colonies) were found in similar proportions in the My-10-positive and control cell populations.

Pure erythroid colonies were enumerated at Day 14 in the same panned marrow cell fractions (methylcellulose-containing cultures, Table 7, 8). Pure erythroid colonies were several-fold enriched in the My-10-positive fraction, but some erythroid colonies were also present in the My-10-negative cell populations. It was noted that all of the large (more than 200 cells) erythroid colonies with the microscopic characteristics of BFU-E (multiple hemoglobinized clusters of cells forming a large colony) were My-10-positive. Though small (less than 200 cell) erythroid colonies (enumerated on day 14, but with the morphology of CFU-E in that they were composed of only a single cluster of hemoglobinized cells) were enriched in the My-10-bound fraction, substantial numbers of small erythroid colonies were My-10-bound.

Smaller numbers of pure eosinophilic colonies were observed in these methylcellulose-containing marrow cultures. The pure eosinophilic colonies (CFC-Eo) were depleted in the My-10-negative fraction and enriched in the My-10-positive fraction (Table 7, 8). Over 80% of CFC-Eo were My-10-positive by this methodology. Even smaller numbers of mixed eosinophilic-erythroid colonies (CFC-EEo) were observed, all in the My-10-positive cell population (Table 7, 8).

TABLE 6

CFC-GM (agar cultures) in My-10-Panned Normal Human Marrow Cells

	MOPC 21		My-10	
	Unbound	Bound	Unbound	Bound
A. Single Experiment:				
Recovered Viable Cells*	84%	1%	77%	3%
CFC-GM per 10^5 Cells**	63(±5)****	ND	2(±1)	883(±69)
Recovered CFC-GM***	5290	ND	192	2650
B. Averaged Data: (9 experiments)				
Recovered Viable Cells	83(±2)%	3(±1)%	81(±1)%	6(±1)%
CFC-GM Enrichment	[1]	0	0.08(±0.02)	8(±2)
CFC-GM	[100%]	0%	9(±3)%	42(±5)%

blast cells were My-10-positive (by either panning or immune rosetting).

TABLE 5

Anti-My-10 Immune Rosetting-Human Marrow Cells: Differential Nucleated Cell Counts*

	Anti-My-10-Rosetting Marrow Cells**		
	Direct Assay	Indirect Assay	Whole Marrow
Blast Cells	48%	63%	10%
Promyelocytes	11	8	10
Myelocytes	0	0	13
Metamyelocytes	0	1	16
Band forms and segmented neutrophils	11	8	18
Monocytes	0	0	15
Lymphocytes	8	1	10
Orthochromatophilic normoblasts	2	2	6
Polychromatophilic normoblasts	0	0	3
Unidentifiable***	20	17	0

*Based upon 200 cell counts (rounded). Whole marrow was taken as the first 200 nucleated cells seen on the indirect anti-My-10 test slide, whether rosetted or not.
**In wet mounts from this experiment, 1.5% of marrow cells formed direct anti-My-10 rosettes, and 3.0% formed indirect anti-My-10 rosettes. Comparison results using MOPC 21 control rosettes were 0% (direct and indirect), and using 2B/43/6 rosettes were 100% (direct and indirect).
***Morphology obscured by rosetted erythrocytes.

EXAMPLE V

Expression of My-10 by Human Myeloid Colony-Forming Cells for Granulocytes and Monocytes (CFC-GM)

Normal marrow cell fractions obtained as above were assayed for CFC-GM in semisolid agar cultures.

Day 12-14 CFC-GM were assayed in triplicate in semi-solid agar with 5% placenta-conditioned medium (Pike and Robinson, (1970) *J. Cell. Physiol.* 76:77; Burgess et al., (1977) *Blood* 49:573) exactly as described previously (Strauss et al., (1983) *Blood* 61: 1222). Day 14 multilineage colonies (Fauser and Messner, (1979) *Blood* 53:1023; Nakahata et al., (1982) *Blood* 59:857; Iscove, et al., (1974) *J. Cell. Physiol.* 83:309) were assayed in quadruplicate in medium containing 0.96% methylcellulose, 5% placenta-conditioned medium, and 1 unit/ml erythropoietin (Connaught, Torronto, ONT). Colony number was a linear function of total cells plated. It should be noted that, in most experiments, cells were plated at several dilutions to obtain countable plates (20-200 colonies). This was particularly important with My-10-positive cell fractions, which were enriched in colony-forming cells. In addition, mixed lineage colonies were not scored on plates with more than 100 total colonies per plate, to avoid scoring superimposed colonies as products of a single colony-forming cell.

Colonies were counted in situ using a dissecting microscope (50-80×) or inverted phase microscope (200×), and gross colony and cellular morphology was recorded. Representative colonies were plucked using a Pasteur pipette. Stained cytocentrifuge preparations were analyzed for confirmation of cell type(s) within the colonies.

Less than 10% of the CFC-GM were detected in the My-10-negative cell fraction, and the My-10-positive cell fraction was several-fold enriched for CFC-GM, compared to unfractionated marrow or control IgG1 (MOPC 21)-bound marrow cells (Table 6). However, only approximately 40% of the CFC-GM of the initial marrow sample were recovered in the My-10-positive cell population. This might be explained by mechanical injury to the My-10-positive cells or by partition of an accessory cell type Sharkis et al., (1981) In Gershwin and Merchant (eds), *Immunologic Defects in Laboratory Animals* (Plenum, NY) 1:79; Strauss et al., (1983) *Blood*, in press).

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CFC-GM	[1]	0	0.08(±0.02)	8(±2)
Enrichment CFC-GM	[100%]	0%	9(±3)%	42(±5)%

TABLE 6-continued

CFC-GM (agar cultures) in My-10-Panned Normal Human Marrow Cells				
	MOPC 21		My-10	
	Unbound	Bound	Unbound	Bound
Recovery				

*Values represent (100%) × (viable cell number in fraction)/(initial cell number treated with McAb).
**Arithmetic mean (± standard deviation) of at least triplicate determinations (rounded).
***Product of (CFC-GM/10^5 cells) × (number of viable cells in fraction).
****Similar results obtained with unfractioned marrow cells.
Not done.
(CFC-GM per 10^5 cells in given cell fraction)/(CFC-GM per 10^5 cells in MOPC-21-unbound fraction for that experiment). Mean ± 1 standard error of the mean (SEM).
(100%) × (Recovered CFC-GM in given fraction)/(Recovered CFC-GM in MOPC-21-unbound fraction for that experiment). Mean ± 1 SEM.
The MOPC-21-bound fraction was large enough to permit plating for CFC-GM on only 2 of these 9 experiments. In both of these experiments and in 6 additional experiments, no CFC-GM colonies grew on the plates.

TABLE 7

Colonies in Methylcellulose Culture After Panning (single experiment)				
	MOPC 21		My-10	
	Unbound	Bound	Unbound	Bound
Recovered Viable Cells*:	84%	1%	77%	3%
Large CFC-GM:				
Per 10^5 Cells**	57(±6)	ND	1(±1)	1600(±180)
Recovery***	4790	ND	77	4800
Small CFC-GM:				
Per 10^5 Cells	106(±17)	ND	5(±2)	3650(±400)
Recovery	8900	ND	385	10950
Large Erythroid:				
Per 10^5 Cells	58(±2)	ND	0(±0)	1450(±200)
Recovery	4870	ND	0	4350
Small Erythroid:				
Per 10^5 Cells	142(±2)	ND	94(±12)	1970(±490)
Recovery	11930	ND	7240	5850
CFC-EEo:				
Per 10^5 Cells	2(±1)	ND	0(±0)	50(±0)
Recovery	168	ND	0	150
CFC-Eo:				
Per 10^5 Cells	14(±2)	ND	3(±1)	380(±150)
Recovery	1090	ND	231	1200

*, **, ***Same as Table 6.
Large colonies contained at least 200 cells, small colonies less than 200 cells.
Not done.
CFC-Eo = pure eosinophil colonies. CFC-EEo = mixed colonies of the CFC-GEMM-type containing erythrocytes and eosinophils.

TABLE 8

Percent of Colonies in My-10-Bound Marrow Cell Panning Experiments*	
	Percent Recovered in My-10 Bound Fraction
Viable Cells	4(±1)%
Large CFC-GM	95(±2)
Small CFC-GM	84(±8)
Large Erythroid	78(±10)
Small CFC-GM	46(±14)
CFC-EEo	98(±2)
CFC-Eo	86(±6)

*Arithmetic means (rounded ± 1SEM) in 4 experiments plated in methylcellulose cultures. Definitions of colony types, etc., as in Table 7.

EXAMPLE VI

FACS II Sorting of My-10-Treated Marrow Cells

Under aseptic conditions, normal low density, nonadherent marrow cells were incubated with centrifuged anti-My-10, washed, then reacted with centrifuged, fluorescein-conjugated, anti-mouse IgG (as above for analytical indirect immunofluorescence). After washing, the cells were analyzed and sorted on the basis of fluorescence intensity (FACS II). "My-10-bright" cells were defined as more than 50 channels fluorescence intensity (1.93% of total My-10-treated cells; in contrast, 0.05% of the MOPC-21-treated cells were brighter than 50 channel units). The FACS II was adjusted to deflect anti-My-10-treated cells with fluorescence intensity less than 30 channels into the "My-10-dull" fraction (97.14% of total sorted cells). A "window" of cells between 30–50 channels fluorescence intensity (0.93% of total My-10-treated cells) was discarded to minimize overlap. The My-10-bright fraction consisted almost entirely of morphologically-defined blast cells (Table 9) Cytochemical assays suggested that the FACS-separated My-10-positive blast cells were heterogeneous, containing at least monoblasts and myeloblasts (confirming cytochemical studies on panned My-10-positive cells).

The My-10-positive fraction contained essentially all of the colony-forming cells, and was more than 50-fold enriched for these progenitor cell types (Table 10). 18% of the My-10-positive cells formed colonies detectable in this culture system. These FACS results are in agreement with the results using the panning methodology, except that FACS apparently yielded a population of My-10-positive cells that was more enriched in primitive and clonogenic cells.

TABLE 9

Cytochemical Analysis of FACS-Separated My-10-Antigen-Positive Primitive Cells*	
Cytochemical Stain	Percent Primitive Cells Cytochemically Positive**
Peroxidase	14%
Sudan Black	10
Periodic Acid Schiff	16
NASD Chloroacetate Esterase	8
Nonspecific Esterase Diffusely Stained	28***
Focally Stained	1***

*3% of the FACS-Separated My-10-antigen-positive cells were matured neutrophils (metamyelocytes, band forms, segmented neutrophils), 6% were mature monocytes, and 1% were mature lymphocytes. These mature cells were not scored in this analysis of the "primitive blast" cells (84%, all morphologically immature with a fine, open chromatin pattern) and promyelocytes (6%).
**200 cells counted; each cytochemical test was done on a separate slide, except for the esterases which were done on the same slide.
***Values were zero with NaF added (NaF inhibits nonspecific esterase of monocytes).

TABLE 10

Colonies in Methylcellulose Culture After FACS Experiment			
	Unsorted*	My-10-Dull	My-10-Bright
Recovered Viable Cells:	[100%]	97%	2%
Colonies per 10^5 Cells**:			
Large CFC-GM	50(±21)	0(±0)	4150(±680)
Small CFC-GM	147(±41)	2(±0)	7750(±1980)
Large Erythroid	9(±1)	0(±0)	1800(±690)
Small Erythroid	52(±6)	4(±1)	3400(±910)
Eosinophil-containing	11(±7)	0(±0)	550(±380)

*Cells were anti-My-10-treated and passed through FACS laser, but not sorted.
**Definitions of methylcellulose culture CFC-GM and erythroid colonies as described in text and previous Tables. Eosinophil-containing colonies include CFC-Eo and CFC-EEo. Low erythroid colony growth was observed in this experiment.

EXAMPLE VII

Immunoprecipitation of a Radiolabelled KG-1*a* Antigen by Anti-My-10

Vectorial labelling of the plasma membrane of intact cells with 125 I-iodide, followed by immunoprecipitation with SA-bound monoclonal antibody, SDS-PAGE analysis, and visualization of antigen by autoradiography, was utilized to identify the KG-1*a* membrane protein detected by anti-My-10. Under reducing as well as non-reducing conditions, My-10 antigen had an Mr of approximately 115 kD, indicating the absence of disulfide-linked oligomers.

KG-1*a* cells were radiolabelled vectorially within 125 I-iodide using the method of Hubbard and Cohn ((1972) *J. Cell Biol.* 55:390). Briefly, 20 million cells in exponential growth were washed four times in 10 mM Hepes—0.15 M NaCl buffer, pH 7.4 (Buffer A). The cell pellet was resuspended in one ml of Buffer A containing 0.05 M glucose, 40 ul of (100 IU/ml) lactoperoxidase (Calbiochem-Behring, San Diego, CA), and 2.5 ul of freshly prepared (1 mg/ml) glucose oxidase (Millipore Corp., Freehold, NJ). 0.5–1 mCi of 125 I-iodide (New England Nuclear, Boston, MA) was added, and the cell suspension was incubated at 22° C. for 20 minutes with gentle agitation. Then 10 ml of Buffer A containing 4 mM KI and 0.1% glucose was added to stop the reaction. After four washes with Buffer A, the cell pellet was resuspended in 500 ul of disruption buffer (10 mM EDTA, and 50 ug/ml Leupeptin [Sigma]) for 20 minutes on ice with periodic vortexing. The cell extract was then centrifuged (10 minutes, 15,600×g, 4° C.), and the supernate used for immunoprecipitation.

Immunoprecipitation was performed essentially as described by Lampson, in *Monoclonal Antibodies* 395–397 (Kennett, et al. 1980). For each monoclonal antibody to be tested, 300 ul of 10% fixed, whole, protein A-bearing Cowan strain Staphylococci (SA; Calbiochem-Behring) was washed three times by centrifugation (15,600×g, 5 min., 4° C.) in Lampson wash buffer (WB) (0.1M phosphate-buffer saline, pH 8.6, containing 0.1% BSA, 0.02% NaN3, 0.5% NP40, 0.1% SDS). The SA pellet was then resuspended to the initial volume with goat anti-mouse IgG serum (Kierkegaard and Perry, Gaithersburg, MD) and incubated 12–18 hrs. at 4° C. The SA-IgG complex was washed seven times in WB and suspended with monoclonal antibody (hybridoma culture supernate) to 10% (v/v). After 40 minutes incubation (22° C.), the SA-IgG-monoclonal antibody complex was washed three times in WB and resuspended to the initial volume in WB. To this complex, 80–120 ul of cell extract was added, followed by incubation at 4° C. for 12–18 hours. The SA-IgG-monoclonal antibody complex was then washed three times in WB and resuspended in 50 ul of WB plus 25 ul of Laemmli ((1970) *Nature* 227:680) sample buffer (0.0625M Tris HCl, pH 6.8, containing 12.5% glycerol, 1.25% 2-mercaptoethanol, 5% SDS and 1 mM EDTA), boiled for two minutes, centrifuged (15,600×g, 5 min.), and the supernate harvested for analysis by SDS-polyacrylamide gel electrophoresis.

The samples were analyzed on 10% SDS-polyacrylamide gels under reducing conditions according to the method of Laemmli ((1970) *Nature* 227:680). After electrophoresis, the gel was stained with Coomassie brilliant blue, destained, dried onto filter paper and exposed to X-ray AR film (Kodak, Rochester, NY) at −70° C.

EXAMPLE VIII

Reactivity of Anti-My-10 with Diagnostic Specimens from Patients with Acute Leukemia

Initial diagnostic marrow specimens from Johns Hopkins Oncology Center patients found to have leukemia, with at least 80% marrow blast cells, were tested with these antibodies by indirect immunofluorescence. Specimens which contained at least 20% fluorescent cells (over background) were counted as "positive" for that antigen (Strauss et al., (1983) *Blood*, in press). The My-10 antigen was expressed on blast cells from approximately 30% of the acute leukemia specimens, both lymphocytic and nonlymphocytic, but on none of the few chronic leukemia specimens tested, including two specimens of chronic myelogenous leukemia (CML) in "myeloid" blast crisis or other specimens tested (Table 11).

TABLE 11

Reactivity of Patients' Marrow* Leukemic Blast Cells With Anti-My-10

Disease**		Percent Positive Specimens***
Acute Nonlymphocytic Leukemia		28% (18/65)
Acute Lymphocytic Leukemia		32% (10/31)
cALLa-positive	(8/23)	
HLA-DR-positive, cALLa-negative	(2/3)	
T-cell (leu-1 or T11-positive)	(0/5)	
Chronic Lymphocytic Leukemia		0% (0/10)
Chronic Myelogenous Leukemia		0% (0/3)
Myeloblastic crisis	(0/1)	
Basophilic blast crisis	(0/1)	
Untreated chronic phase	(0/1)	
Mycosis fungoides**		0% (0/1)
Lymphoma Non-T, non-B	(0/1)	0% (0/2)
B-cell	(0/1)	
Undifferentiated carcinoma (marrow involvement)		0% (0/1)

*Peripheral blood (at least 80% leukemic mononuclear cells) was studied instead of bone marrow in 9 chronic lymphocytic and 3 acute lymphocytic leukemia specimens as well as in the 1 mycosis fungoides specimen. Ascites cells or mechanically dissociated cells from lymphomatous nodes were studied in the 2 patients with lymphoma.

**Diagnosis defined by clinical features, blast cytomorphology and cytochemistry, and immunologic markers. See Nadler et al., Diagnosis and Treatment of Human Leukemias and Lymphomas Utilizing Monoclonal Antibodies, pp. 187-225 (E. Brown 1981).

***Values represent percent of specimens with at least 20% (above MOPC 21 background) antibody-labelled cells (number positive specimens/number tested).

Since variations will be apparent to those skilled in the art, it is intended that this invention be limited only by the scope of the appended claims.

I claim:

1. A monoclonal antibody which specifically binds to an antigen on non-malignant, immature human marrow cells, wherein said antigen is stage specific and not lineage dependent, and said antigen is also specifically bound by the antibody produced by the hybridoma deposited under ATCC Accession No. HB-8483;

(a) which antigen is present on non-malignant, human blood or bone marrow:

(i) colony-forming cells for granulocytes and monocytes (CFC-GM),

(ii) colony-forming cells for erythrocytes (BFU-E),

(iii) colony-forming cells for eosinophils (CFC-Eo),

(iv) multipotent colony-forming cells (CFC-GEMM), and

(v) immature lymphoid precursor cells;

(b) which antigen is present on a maximum of about 5% non-malignant, human marrow cells and a max-

imum of about 1% non-malignant, human peripheral blood cells; and

(c) which antigen is not present on non-malignant, mature human myeloid and lymphoid cells.

2. The monoclonal antibody of claim 1 that corresponds to the monoclonal antibody produced by the hybridoma deposited under ATCC Accession No. HB-8483.

3. The monoclonal antibody of claim 1 that is the antibody produced by the hybridoma deposited under ATCC Accession No. HB-8483.

4. A hydbridoma which produces a monoclonal antibody of claim 1.

5. A hybridoma which produces a monoclonal antibody of claim 2.

6. The hybridoma deposited under ATCC Accession No. HB-8483.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 4,965,204
DATED : October 23, 1990
INVENTOR(S) : Curt I. CIVIN

It is certified that error appears in the above-indentified patent and that said Letters Patent is hereby corrected as shown below:

Column 20, line 56 (line 6 of claim 1), delete all the text of claim 1 after "HB-8483".

Column 21, line 6, after the word "that", delete "corresponds to" and insert --specifically binds a binding site on said antigen which overlaps the binding site on said antigen specifically bound by--

Signed and Sealed this
First Day of August, 1995

Bruce Lehman

Attest:

Attesting Officer

BRUCE LEHMAN
Commissioner of Patents and Trademarks

[redacted]

UNITED STATES PATENT AND TRADEMARK OFFICE

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DATED : October 23, 1990
INVENTOR(S) : Curt I. CIVIN

It is certified that error appears in the above-indentified patent and that said Letters Patent is hereby corrected as shown below:

Column 1, line 8 , please add:

--The invention described herein was made in the course of work under a grant or award from The Department of Health and Human Services.--

Signed and Sealed this

Seventh Day of October, 1997

Attest:

BRUCE LEHMAN

Attesting Officer

Commissioner of Patents and Trademarks

Exhibit D

UNITED STATES PATENT AND TRADEMARK OFFICE

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Exhibit E

DEPARTMENT OF HEALTH & HUMAN SERVICES
Public Health Service

Food and Drug Administration
1401 Rockville Pike
Rockville, MD 20852-1448

OIPE JC
AUG 31 1999
PATENT & TRADEMARK OFC

July 2, 1999

Tung Koh
Director, Regulatory Affairs
Nexell Therapeutics, Inc.
9 Parker
Irvine, CA 92718-1605

Re:	BP 97-0001
Product:	Isolex 300 Magnetic Cell Selection System
Filed:	February 24, 1997
Amended:	See appended list
Re:	BP 97-0001/01
Product:	Isolex 300i Magnetic Cell Selection System
Filed:	February 3, 1998
Amended:	See appended list

Dear Ms. Koh:

The Center for Biologics Evaluation and Research (CBER) of the Food and Drug Administration (FDA) has completed its review of your premarket approval application (PMA) and supplement for the Isolex 300 Magnetic Cell Selection System and for the Isolex 300i Magnetic Cell Selection System, respectively. These devices are indicated for processing autologous peripheral blood progenitor cell (PBPC) products to obtain a CD 34+ cell enriched population intended for hematopoietic reconstitution after myeloablative therapy in patients with CD 34-negative tumors. We are pleased to inform you that the PMA and supplement are approved subject to the conditions described below and in the "Conditions of Approval" (enclosed here and previously communicated to you in a letter dated January 7, 1999). You may begin commercial distribution of the device upon receipt of this letter.

The sale, distribution, and use of this device are restricted to prescription use in accordance with 21 CFR 801.109 within the meaning of section 520(e) of the Federal Food, Drug, and Cosmetic Act (the act) under the authority of section 515(d)(1)(B)(ii) of the act. FDA has also determined that to ensure the safe and effective use of the device, the device is further restricted within the meaning

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of section 520(e) under the authority of section 515(d)(1)(B)(ii), (1) insofar as the labeling specify the requirements that apply to the training of practitioners who may use the device as approved in this order; and (2) insofar as the sale, distribution, and use must not violate sections 502(q) and (r) of the act.

In addition to the post-approval requirements in the enclosure, the post-approval reports must include the following information:

1. You have agreed to conduct the post-marketing studies detailed below. An outline of the protocols for these studies are to be provided to CBER within three weeks of receipt of this letter to be followed, within 3 months, by more detailed protocols and plans for implementation, including timelines for completion.

a. Laboratory Studies:

i. Full scale studies using apheresis products to determine the effect of leukocyte counts, total nucleated cell counts and platelet counts on yield, purity and viability of CD34+ selected cells. These studies will be designed to determine the level (absolute number, relative percentage) of granulocytes, platelets, and/or other cellular elements which will result in a higher risk/incidence of performance failure.

ii. Full scale studies using apheresis products to determine the effects of anti-coagulant concentration (ACD-A to cell ratio) and the composition of suspension and wash solutions used for prior apheresis collection on yield, purity and viability of CD34+ selected cells and to assess factors which will result in a higher incidence of performance failure.

iii. Full scale studies using apheresis products to determine the effect of paraprotein levels, over a range expected to be observed in subjects with multiple myeloma and other monoclonal gammopathies, on yield, purity and viability of CD34+ selected cells.

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iv. Full scale studies using apheresis products to determine the effect of prior freezing and thawing on yield, purity and viability of CD34+ selected cells.

v. Full scale studies using apheresis products to explore approaches for maximum recovery of CD34+ cells from the non-target cell collection under conditions of separation/selection failure.

b. Surveillance Study:

Provide a plan for a surveillance study for performance failures for each device which will capture critical information using case report forms (CRFs) and describe suitable follow-up measures. This reporting study should capture data on the specific causes of performance failures or problems, including but not limited to, the contribution of all of the above factors (see laboratory studies). The reporting period should be the first three years post-approval, and, once the data are analyzed, to discuss with the FDA whether the study should be continued or closed.

The CRFs should capture information regarding the absolute number and relative percentage of: total nucleated cells, granulocytes, and platelets in the apheresis product, and the yield of CD34+ cells after selection; ACD-A anticoagulant to cell ratio; presence (and if present immunoglobulin subtype and serum level) of paraproteins; and information regarding cryopreservation of the cellular products prior to selection. The plan for evaluation of performance failures and CRFs are to be provided within three weeks of the receipt of this letter.

2. As stated in your commitment dated June 22, 1999, we acknowledge that you will:

a. improve the information contained in the lot release certificates of analysis, specifically: to include a specification for pH range for the Dynabeads M-450 Sheep Anti-Mouse IgG and to include the actual values obtained for the LAL tests for each component of the Reagent Kit;

b. include two additional lots of the Anti-CD34-Monoclonal Antibody in your ongoing stability studies;

c. establish a limit for aggregates observed during the SDS-PAGE analysis of the Anti-CD34-Monoclonal Antibody;

d. monitor the temperature in shipments of Dynabeads-M-450 Sheep Anti Mouse IgG from Norway to California by inclusion of a temperature recording device;

We acknowledge your commitment dated June 22, 1999, to assure that all outstanding issues associated with the inspection of your production facilities are satisfactorily resolved and corrective actions completed.

Expiration dating for the components of the Reagent Kit has been established and approved as follows: at 18 months for the Anti-CD34-Monoclonal Antibody (murine) when stored at 2-8°C; at 30 months for the Dynabeads M-450 Sheep Anti-Mouse IgG when stored at 2-8°C; at 36 months for the PR 34+ Stem Cell Releasing Agent when stored at 2-8°C. The dating period for the disposable sets has been established and approved at 24 months when stored at ambient temperature. This is to advise you that, with the exception of the Anti-CD34-Monoclonal Antibody, the protocols you used to establish these expiration dating periods are considered approved protocols for the purpose of extending the expiration dating as provided by 21 CFR 814.39(a)(8).

CBER will publish a notice of its decision to approve your PMA in the FEDERAL REGISTER. The notice will state that a summary of the safety and effectiveness data upon which the approval is based is available to the public upon request. Within 30 days of publication of the notice of approval in the FEDERAL REGISTER, any interested person may seek review of this decision by requesting an opportunity for administrative review, either through a hearing or review by an independent advisory committee, under section 515(g) of the Federal Food, Drug, and Cosmetic Act (the act).

Failure to comply with the conditions of approval invalidates this approval order. Commercial distribution of a device that is not in compliance with these conditions is a violation of the act.

CONFIDENTIAL

Page 5 - Ms. Koh

You are reminded that as soon as possible, and before commercial distribution of your device, that you must submit an amendment to this PMA submission with copies of all approved labeling in final printed form.

All required documents should be submitted in triplicate, unless otherwise specified, to the address below and should reference the above PMA number to facilitate processing.

Document Control Center (HFM-99)
Center for Biologics Evaluation and Research
Food and Drug Administration
1401 Rockville Pike
Rockville, Maryland 20852-1448

If you have any questions concerning this approval order, please contact Terrye G. Zaremba at(301) 827-5103.

Sincerely,

Jay P. Siegel, M.D., FACP
Director
Office of Therapeutics
Research and Review
Center for Biologics
Evaluation and Research

Enclosure

CONFIDENTIAL

U.S. SUBMISSION INDEX

FDA File # Submission Title	IND/IDE Serial #	Sponsor	Submission Date	Approval Date	Comments: Submission Purpose	Comments: Referenced MFs, other Submissions	Comments: Investigators Baxter Protocol #
BP970001 Pre-Market Approval for the Isolex® 300 Magnetic Cell Separation System	000	Nexell	02/21/97		Original submission (23762, etc.)		
	001		03/21/97		Submission of clinical data in SAS file.		
	002		03/25/97		Re-submission of SAS clinical data diskettes by Applied Logics		
	003		04/18/97		Submission of 11/21/96 amendment to DNF 5070 as a PMA amendment (0023997)		
	004		06/30/97		SAS file with current data for the pivotal study that was submitted by ALA on 6/24/97 to FDA directly. This is chiv purposes.		
	005		7/1/97		Response to FDA's 6/18/97 clinical questions		
	006		07/02/97		Draft briefing package for the advisory committee. (24310)		
	007		07/10/97		Final briefing package		
	008		07/15/97		SAS data containing follow-up data for relaspe and death from Applied Logics.		
	009		07/30/97		Response to FDA's 6/27/97 questions (24378)		
	010		08/14/97		Baxter comments to 7/24 Advisory Committee meeting (24530)		
	011		12/16/97		Baxter response to FDA's 11/21/97 Non-Approvable letter, including filling site change from Baxter Hyland to CBL for the anti-Cd34 mAb. (24912)		
	012		01/16/98		Request of extension to respond to FDA 12/11/97 letter for CJD information (25075)		
	013		01/16/98		Notification of change of ownership from Baxter to BIT (25066)		
	014		02/03/98		An extra copy of the Vol. 1 and 3 of PMA amendment 11 for a PCR consultant as requested by Amy Rosenberg on Jan 29, 1998		
	015		02/18/98		Request of extension to reply to the 12/11/97 Dr. Zoon letter for the CJD related information (25167)		

AUG 02 '99 12:17 FR NEXELL THERAPEUTICS 949 588 7570 TO 7196040 P.02

U.S. SUBMISSION INDEX

FDA File # Submission Title	IND/IDE Serial #	Sponsor	Submission Date	Approval Date	Comments		
					Submission Purpose	Referenced MFs, other Submissions	Investigators Baxter Protocol #
	016		02/28/98		Reply to the 12/11/97 Dr. Zoon letter for the CJD related information (25182)		
	017		03/09/98		Notify FDA of company name change Nexell Therapeutics Inc. (25202)		
	018		04/13/98		Day 100 Meeting Request (30048)		
	019		05/04/98		Partial Response to FDA 04/21/98 Questions (30203 & 30196)		
	020		05/05/98		The remaining response to FDA 04/21/98 Questions (30206)		
	021		05/19/98		Correction of tumor depletion data submitted on Dec 17, 1997 (PMA 970001, Amendment 11) and SAS diskette for the tumor depletion data (30271)		
	022		06/04/98		Nexell confirmation of CBER's intent for the May 22, 1998 180 extension of review (30350)		
	023		06/22/98		Authorization for Mike Beatrice to represent Nexell.(30435)		
	024		07/09/98		Agenda for the 7/16 FDA meeting		
	025		07/14/98		Preread package for the 7/16 FDA meeting (30487)		
	027		08/27/98		Response to FDA 7/2/98 Letter and notification of changes to the anti-CD34 mAb appearance spec (30656, 30491, 30679, 30660, 30631 & 30454)		
	028		10/28/98		Response to Amy Rosenberg's Oct 7 request of BIS data listing (92004-300201) and assay validation data from U. of Colorado (DSD-003) for tumor depletion studies. (30877)		
	029		11/20/98		CD ROM Data File for 92004-302103 that includes the format directory. (30932)		
	030		12/01/98		Additional Isolex 300i patient survival follow-up data, clarification of magnet used for the Daudi cell assay, and depletion values for Table 3-1 in the May 1998 Amendment (30943)		

AUG 02 '99 12:17 FR NEXELL THERAPEUTICS 949 588 7570 TO 7196040 P.03

U.S. SUBMISSION INDEX

FDA file # Submission title	IND/IDE Serial #	Sponsor	Submission Date	Approval Date	Comments		
					Submission Purpose	Referenced MFs, other Submissions	Investigators Baxter Protocol #
	031		01/27/99		Submission of revised package insert (31119,31120)		
	032		02/09/99		Response to FDA's 01/07/99 Approvable Letter (31153, 31150 & 31151)		
	033		02/12/99		Additional copy of Vol 2 submitted on 02/09/99 (31167)		
	034		03/29/99		Clarification of the revised Table 7 in the 02/09/99 submission. (31326)		
	035		04/02/99		Acceptance of negotiation of package insert by email (31360)		
	036		04/15/99		Amendment for concurrence of Conditions of Approval defined in the 01/07/99 FDA Letter (31402)		
	037		04/22/99		Draft package insert and copy of the reference for the PI. (31434, 31435)		
	038		05/17/99		Derived SAS data set for CD34, B-cell, T-cell and breast cancer cell for Dr. Misra to support the package insert. (31481)		
	039		06/09/99		Updated stability report for anti-CD34 mAb, peptide, Dynabeads and disposable sets for max dating.(31528)		
	040		06/18/99		Written commitment for the post marketing studies. (31568)		
	041		06/22/99		Written commitment for the post marketing studies for the GMP inspectional issues. (31571)		
	042		06/25/99		New distribution plan (31575)		
	043		06/25/99		Final labeling artwork.		
	044		07/23/99		Post Marketing Studies Plan (31640)		
BP970001/01 Supplemental Pre-Market Approval for the Isolex® 300i Magnetic Cell Separation System	000	Nexell	02/02/98		Original submission of supplement for the Isolex® 300i system. (Cover Letter - 25112; Supplement 25105, Clin Sections 24903, 25097 and 25105)		

AUG 02 '99 12:18 FR NEXELL THERAPEUTICS 949 588 7570 TO 7196040 P.04

U.S. SUBMISSION INDEX

FDA File # Submission Title	IND/IDE Serial #	Sponsor	Submission Date	Approval Date	Comments		
					Submission Purpose	Referenced NFs, other Submissions	Investigators Baxter Protocol #
	001		04/13/98		Day 100 Meeting Request (30048)		
	002		04/24/98		SAS Data Diskette requested by CBER on 04/15/98		
	003		05/19/98		SAS data file and print out for the corrected and updated clinical data (30721)		
	004		06/04/98		Nexell confirmation of CBER's intent for the May 22, 1998 180 extension of review (30350)		
	005		06/09/98		Apply Logics Submission for updated SAS data base to include additional follow-up data for Study 92004-302103		
	006		07/09/98		Agenda for the 7/16/98 FDA meeting.		
	007		07/14/98		Preread package for the 7/16 FDA meeting (30487)		
	009		08/27/98		Response to FDA 7/2/98 Letter and notification of changes to the anti-CD34 mAb appearance spec (30656, 30491, 30679, 30660, 30631 & 30454)		
	010		10/28/98		Response to Amy Rosenberg's Oct 7 request of BIS data listing (92004-300201) and assay validation data from U. of Colorado (DSD-003) for tumor depletion studies. (30877)		
	012		11/20/98		CD Rom Data File for 92004-302103 that includes the format directory. (30932)		
	013		12/01/98		Additional Isolex 300i patient survival follow-up data, clarification of magnet used for the Daudi cell assay, and depletion values for Table 3-1 in the May 1998 Amendment (30943)		
	014		01/27/99		Submission of revised package insert (31119,31120)		
	015		02/09/99		Response to FDA's 01/07/99 Approvable Letter (31153, 31150 & 31151)		
	016		02/12/99		Additional copy of Vol 2 submitted on 02/09/99 (31167)		
	017		03/29/99		Clarification of the revised Table 7 in the 02/09/99 submission. (31326)		

AUG 02 '99 12:18 FR NEXELL THERAPEUTICS 949 588 7570 TO 7196040 P.05

U.S. SUBMISSION INDEX

FDA File # Submission Title	IND/IDE Serial #	Sponsor	Submission Date	Approval Date	Comments		
					Submission Purpose	Referenced MFs, other Submissions	Investigators Baxter Protocol #
	018		04/2/99		Acceptance of negotiation of package insert by email (31360)		
	019		04/15/99		Amendment for concurrence of Conditions of Approval defined in the 01/07/99 FDA Letter (31402)		
	020		04/22/99		Draft package insert and copy of the reference for the PI. (31434, 31435)		
	021		05/17/99		Derived SAS data set for CD34, B-cell, T-cell and breast cancer cell for Dr. Misra to support the package insert. (31481)		
	022		06/09/99		Updated stability report for anti-CD34 mAb, peptide, Dynabeads and disposable sets for max dating. (31528)		
	023		6/18/99		Written commitment for the post marketing studies. (31568)		
	024		6/22/99		Written commitment for the post marketing studies for the GMP inspectional issues. (31571)		
	025		6/25/99		New distribution plan (31575)		
	026		06/25/99		Final labeling artwork.		
	027		07/23/99		PMS Plan (31640)		
BB-IDE 5272 (Autologous) Stem Cell Concentrates (from PBSC) for Hematopoietic Recovery (NHL)	000	Nexell	09/24/93	10/28/93 (verbal) 12/06/93 (written)	Original submission (#9148)	BB-MF 4018 (Dynal) DMF 8236 (Boots) NDA 18-663 (Boots) BB-MF 5134 (Asahi)	Anthony Ho
	001		01/07/94		Add Dr. Gisselbrecht and his protocol (#10456) Entitled "Use of CD34 Cells Isolated with the Baxter Isolex® 300 System for Acceleration of Hematologic Recovery from Dose Chemotherapy in Patients with Advanced Lymphomas." Protocol #4302104 (#10456) Site: Institute d'Hemotologie Principal Investigator: Dr. Christian Gisselbrech		Anthony Ho Gisselbrecht

AUG 02 '99 12:19 FR NEXELL THERAPEUTICS 949 588 7570 TO 7196040 P.06

U.S. SUBMISSION INDEX

FDA File # Submission Title	IND/IDE Serial #	Sponsor	Submission Date	Approval Date	Comments		
					Submission Purpose	Referenced MFs, other Submissions	Investigators Baxter Protocol #
	002		02/25/94		Request a CBER meeting Answer FDA 02/25/94 questions (#1476B)		
	004		03/29/94		Answer FDA 12/06/93 questions (#15564)		
	005		08/09/94		Answer FDA 12/06/93 questions and add Drs. Blaine, Gluckman & Cornetta, protocol entitled "Peripheral Blood Stem Cells (PBSC) or Isolated CD34+ Cells from Mobilized Peripheral Stem Cell Collections for Hematologic Rescue of Advanced Breast Cancer Patients Treated with High-Dose Chemotherapy." Protocol #4302103 Dr. Gluckman and Dr. Cornetta, protocol entitled " Use of Isolex CD34 Cells from Marrow of Natched Related or Unrelated donors for Allogeneic Bone Marrow Transplantation." Protocol #92004 301103 Site: Institute Paoli Calmeto, Marseille, France, Principal Investigator: Dr. Bidier Blaise Hospital Sant Louis, Paris - Principal Investigator - Dr. Elaine Gluckman Indiana University Medical Center - Principal Investigator - Dr. Kenneth Cornetta		
	006		10/14/94		Add Dr. Dreger, Protocol entitled "Use of CD34+ Cells Isolated with the Baxter Isolex 300 System to Purify Peripheral Blood Progenitor Cells for Autotransplantation Following High Dose Chemotherapy for Treatment of Advanced-Stage Low-Grade non-Hodgkin's Lymphoma." Protocol # 92004 302105 (#18284) Site: Bone Marrow Transplant Unit, University of Kiel, Germany - Principal Investigator - Dr. Peter Dreger		
	007		11/18/94		Add Dr. Cornetta, Protocol entitled "PBSC or Isolated CD34+ Cell from Mobolized Peripheral Stem Cell Collections for Hematologic Rescue of Advanced Breast Cancer Patients Treated with High-Dose Chemotherapy." Protocol #4302103 (#18460) Site: Indiana University Medical Center - Principal Investigator - Dr. Kenneth Cornetta		

AUG 02 '99 12:19 FR NEXELL THERAPEUTICS 949 588 7570 TO 7196040 P.07

U.S. SUBMISSION INDEX

FOA File # Submission Title	IND/IDE Serial #	Sponsor	Submission Date	Approval Date	Comments		
					Submission Purpose	Referenced MFs, other Submissions	Investigators Baxter Protocol #
	010		12/21/94		Add Dr. Mark Shlomchik and new site Protocol entitled "PBSC or Isolated CD34+ Cell from Mobilized Peripheral Stem Cell Collections for Hematologic Rescue of Advanced Breast Cancer Patients Treated with High-Dose Chemotherapy." Protocol #4302103 (#18729) Site: Yale University School of Medicine - Principal Investigator - Dr. Mark Shlomchik		
	011		12/21/94		Add a co-investigator (Dr. Mark Adler) (#18730)		
	013		2/14/95		Revised protocol from "mini-beam" to B-cell for Dr. Richard Champlin. Protocol entitled "CD34+ Cells from Mobilized Peripheral Stem Cell Collections for Hematologic Rescue of Patients Treated with High-Dose Chemo/Radiotherapy for B-Cell Malignancies." Protocol #4302104-A (#19027) Site: MD Anderson Cancer Center, Houston, TX		
	014		2/24/95		Annual Report (#19207)		
	015		3/3/95		Add Dr. Jean Lotz Protocol entitled "Peripheral Blood Stem Cells (PBSC) or Isolated CD34' Cells from Mobilized Peripheral Stem Cell Collections for Hematologic Rescue of Advanced Breast Cancer Patients Treated with High-Dose Chemotherapy." Protocol #92004 302103 (#19289) Site: Hospital Tenon Paris, France		Jean-Pierre Lotz
	016		3/8/95		Revised 1571 for #013 - changed from revised protocol to new protocol (#19334)		
	018		3/13/95		Add Dr. Kenneth Cornetta Protocol entitled "CD34+ Cells from Mobilized Peripheral Stem Cell Collections for Hematologic Rescue of Patients Treated with High-Dose Chemo/Radiotherapy for B-Cell Malignancies." Protocol #4302104-A (#19373) Site: Indiana University Medical Center		

AUG 02 '99 12:19 FR NEXELL THERAPEUTICS 949 588 7570 TO 7196040 P.08

U.S. SUBMISSION INDEX

FDA File # Submission Title	IND/IDE Serial #	Sponsor	Submission Date	Approval Date	Comments		
					Submission Purpose	Referenced MFs, other Submissions	Investigators Baxter Protocol #
	019		3/28/95		Add Dr. Randolph Broun Protocol entitled "CD34+ Cells from Mobilized Peripheral Stem Cell Collections for Hematologic Rescue of Patients Treated with High-Dose Chemo/Radiotherapy for B-Cell Malignancies." Protocol #4302104-A (#19479) Site: Saint Louis University - Principal		
	020		3/29/95		Add Dr. Arturo Molina Protocol entitled "CD34+ Cells from Mobilized Peripheral Stem Cell Collections for Hematologic Rescue of Patients Treated with High-Dose Chemo/Radiotherapy for B-Cell Malignancies." Protocol #4302104-A (#19553) Site: City of Hope Medical Center		
	021		3/31/95		Add Dr. Jean Paul Fermand Protocol entitled "CD34+ Cells from Mobilized Peripheral Stem Cell Collections for Hematologic Rescue of Patients Treated with High-Dose Chemo/Radiotherapy for B-Cell Malignancies." Protocol #4302104-A (#19564) Site: Hospital Saint Louis, Paris		
	022		4/5/95		Add 2 new institutional protocols to used with protocol #4302104-A for Dr. Champlin Protocol #1 entitled " CD34' Selection of Mobilized Peripheral Blood Stem Cell Collections for Autologous Transplantation in Patients with Multiple Myeloma Receiving Myeloablative Chemotherapy" Protocol #2 "Intensive Chemotherapy for Mantle Cell Lymphoma". (#19573) Site: M.D. Anderson Cancer Center, Houston, TX		
	024		4/24/95		Modification to Isolex 300 Reagent Kit & selection process (alternate release) (#19692, 19694)	BB-MF 5070 (Baxter) BB-MF 6000 (Dynal) BB-MF 5340 (Chesapeake) BB-MF 2460 (BioWhittaker)	

AUG 02 '99 12:20 FR NEXELL THERAPEUTICS 949 588 7570 TO 7196040 P.09

U.S. SUBMISSION INDEX

FDA File # Submission Title	IND/IDE Serial #	Sponsor	Submission Date	Approval Date	Comments		
					Submission Purpose	Referenced Mfs./ other Submissions	Investigators Baxter Protocol #
	025		4/26/95		Add Dr. Ian Franklin Protocol #1 entitled "CD34' Cells from Mobilized Peripheral Stem Cell Collections for Hematologic Rescue of Patients Treated with High-Dose Chemo/Radiotherapy for B-Cell Malignancies" Protocol # 92004 302104-A Protocol #2 "Peripheral Blood Stem Cells (PBSC) or Isolated CD34' Cells from Mobilized Peripheral Stem Cell Collections for Hematologic Rescue of Advanced Breast Cancer Patients Treated with High-Dose Chemotherapy." Protocol # 92004 302103 (#19781) Site: Glasgow Royal Infirmary, Scotland		
	026		4/26/95		Add Dr. Scott Rowley Protocol entitled "CD34+ Cells from Mobilized Peripheral Stem Cell Collections for Hematologic Rescue of Patients Treated with High-Dose Chemo/Radiotherapy for B-Cell Malignancies." Protocol #4302104-A (#20895) Site: Fred Hutchinson Cancer Center		
	027		05/09/95		Provide draft toxicology reports for the 9069W peptide (#19890)		
	029				Add Dr. Richard Maziarz Protocol #1 entitled "CD34' Cells from Mobilized Peripheral Stem Cell Collections for Hematologic Rescue of Patients Treated with High-Dose Chemo/Radiotherapy for B-Cell Malignancies." Protocol # 92004 302104-A Protocol #2 "Peripheral Blood Stem Cells (PBSC) or Isolated CD34' Cells from Mobilized Peripheral Stem Cell Collections for Hematologic Rescue of Advanced Breast Cancer Patients Treated with High-Dose Chemotherapy." Protocol # 92004 302103 (#20045) Site: Oregon Health Sciences University		
	030		06/15/95		Add Dr. Craig Rosenfeld Protocol entitled "Peripheral Blood Stem Cells (PBSC) or Isolated CD34' Cells from Mobilized Peripheral Stem Cell Collections for Hematologic Rescue of Advanced Breast Cancer Patients Treated with High-Dose Chemotherapy." Protocol #4302103 (#20109) Site: Texas Oncology, P.A. (TOPA)		

AUG 02 '99 12:20 FR NEXELL THERAPEUTICS 949 588 7570 TO 7196040 P.10

U.S. SUBMISSION INDEX

FDA File # Submission Title	IND/IDE Serial #	Sponsor	Submission Date	Approval Date	Comments		
					Submission Purpose	Referenced NFs, other Submissions	Investigators Baxter Protocol #
IDE 5272	031		6/29/95		Protocol entitled "CD34' Cells from Mobilized Peripheral Stem Cell Collections for Hematologic Rescue of Patients Treated with High-Dose Chemo/Radiotherapy for B-Cell Malignancies." Protocol # 92004 302104-A (#20216) Site: UCSD Medical Center - Principal Investigator - Dr. Anthony Ho		
	037		9/18/95		Add Dr. Guido Tricot Protocol entitled "CD34' Cells from Mobilized Peripheral Stem Cell Collections for Hematologic Rescue of Patients Treated with High-Dose Chemo/Radiotherapy for B-Cell Malignancies." Protocol # 92004 302104-A (#19783) Site: University of Arkansas for Medical Sciences		
	038		9/19/95		Submit FDA Form 1572 & CV's for Dr. Ho (UCSD Cancer Center) to supplement info submitted as #031 (#20897)		
	039		9/28/95		Add Dr.Saul Yanovich Protocol #1 entitled "CD34' Cells from Mobilized Peripheral Stem Cell Collections for Hematologic Rescue of Patients Treated with High-Dose Chemo/Radiotherapy for B-Cell Malignancies." Protocol # 92004 302104-A Protocol #2 " Use of Isolex CD34 Cells from Narrow of Matched Related or Unrelated donors for Allogeneic Bone Marrow Transplantation." Protocol #92004 301103 (#21053) Site: Virginia Commonwealth Univ.		
	040		10/5/95		Letter to CBER requesting meeting to discuss Phase III clinical trials for IDE 5272 and IDE 5714.		
	042		11/8/95		Addition of Dr. Skikne Protocol entitled "CD34+ Cells from Mobilized Peripheral Stem Cell Collections for Hematologic Rescue of Patients Treated with High-Dose Chemo/Radiotherapy for B-Cell Malignancies." Protocol #4302104-A (#21313) Site: University of Kansas Cancer Research Center		

AUG 02 '99 12:21 FR NEXELL THERAPEUTICS 949 588 7570 TO 7196040 P.11

U.S. SUBMISSION INDEX

FDA File # Submission Title	IND/IDE Serial #	Sponsor	Submission Date	Approval Date	Comments		
					Submission Purpose	Referenced NFs, other Submissions	Investigators Baxter Protocol #
	043		11/8/95		Submission of Institutional Protocol Amendment for Dr. Ho at UCSD for the B-cell study. (21315)		
	044		11/8/95		Amendment to Dr. Broun's protocol and Informed Consent for study #92004 302104-A (21322)		
	045		12/6/95		Addition of Dr. Stephen Glück Protocol entitled "CD34+ Cells from Mobilized Peripheral Stem Cell Collections for Hematologic Rescue of Patients Treated with High-Dose Chemo/Radiotherapy for B-Cell Malignancies." Protocol #92004 302104-A (#21422) Site: Northeastern Ontario Cancer Center, Sudbury Ontario, Canada		
	046		12/29/95		Amendment to phase-in the Isolex 300i (21442)		
IDE 5272 (cont.)	047		1/8/96		Submission of institutional protocol changes for University of Arkansas (Dr. Tricot) (21491)		
	048		1/8/96		Submission of new sub-investigators (Dr. Tom Chauncey and Dr. Paul Weiden) with Dr. Scott Rowley (FHCRC) and institutional protocol changes. (21562)		
IDE 5272	049		1/10/96		Submission of Ethics Committee approvals for Drs. Gisselbrecht and Fermand for using the stem cell releasing agent for the B-cell protocols. Protocol entitled "CD34+ Cells from Mobilized Peripheral Stem Cell Collections for Hematologic Rescue of Patients Treated with High-Dose Chemo/Radiotherapy for B-Cell Malignancies." Protocol #92004 302104-A (#21570) Site: Hospital of St. Louis, Paris		
	050		1/10/96		Notification of new Principal Investigator for B-cell protocol at Indiana University (Dr. Rafat Abonour replacing Dr. Cornetta) Protocol entitled "CD34+ Cells from Mobilized Peripheral Stem Cell Collections for Hematologic Rescue of Patients Treated with High-Dose Chemo/Radiotherapy for B-Cell Malignancies." Protocol #92004 302104-A (#21571) Site: Indiana University Medical Center		

AUG 02 '99 12:21 FR NEXELL THERAPEUTICS 949 588 7570 TO 7196040 P.12

U.S. SUBMISSION INDEX

FDA File # Submission Title	IND/IDE Serial #	Sponsor	Submission Date	Approval Date	Comments		
					Submission Purpose	Referenced MFs, other Submissions	Investigators Baxter Protocol #
IDE 5272 (cont.)	052		2/8/96		Submission of amended Breast Cancer protocol, #92004 302103. (21776)		
	055		3/12/96		Submission of protocol change for 300sa to 300i for protocol #92004 302103 only for Dr. Shlomchik at Yale New Haven Hospital.		
	056		3/12/96		Submission of protocol change to use Isolex 300i for Dr. Rosenfeld, TOPA. (22029)		
	059		3/14/96		Letter to FDA requesting a Pre-Phase III meeting to review a proposed breast cancer Phase III autologous transplantation study w/Isolex CD34+ selected PBSC & the appropriate clinical end-point; protocol #92004-302301 for BB-IDE 5272. (22084)		
	070		4/5/96		Submission of protocol change from 60 to 200 pts for protocol #92004 302104-A and from 30 to 180 for protocol #92004 302103.		
	071		4/9/96		IDE 5272 Annual Report (22266)		
	072		4/19/96		Letter to Betty Shaw @ FDA regarding the upcoming Pre-Phase III Meeting May 9, 1996 in DC from 1-2:30 p.m. (#22331)		
	076		5/16/96		Submission of IRB approval of protocol change to use Isolex 300i for Dr. Rafat Abnour, Indiana University Medical Center for protocol 92004 302104-A and for Dr. Ken Cornetta for protocol 92004 302103. (22489)		
	077		5/16/96		Submission of IRB approval of protocol change to use Isolex 300i for Dr. Randy Broun at St. Louis Universtiy for protocol 92004 302104-A (22496)		
	078		5/16/96		Submission of IRB approval of protocol change to use Isolex 300i for Dr. Barry Skikne at University of Kansas Medical Center for protocol 92004 302104-A (22497)		

AUG 02 '99 12:21 FR NEXELL THERAPEUTICS 949 588 7570 TO 7196040 P.13

U.S. SUBMISSION INDEX

FDA File # Submission Title	IND/IDE Serial #	Sponsor	Submission Date	Approval Date	Comments		
					Submission Purpose	Referenced MFs, other Submissions	Investigators Baxter Protocol #
	080		5/20/96		The purpose of the current submission (BB-IDE 5272, Amendment 080) is to amend this IDE for: 1) upgrades made to the Isolex 300i instrument on the software (from version 1.10 to 1.11) and hardware, 2) minor design changes made to the Isolex 300i disposable set, and 3) cross-referencing of a Type II Master File on the PR34+™4 Stem Cell Releasing Agent prepared by its supplier. (#22457) Site: Bachem's BB-MF-6465	Bachem's BB-MF-6465	
IDE 5272 (cont.)	083		7/16/96		Add Dr. Diane Krause Protocol entitled "CD34+ Cells from Mobilized Peripheral Stem Cell Collections for Hematologic Rescue of Patients Treated with High-Dose Chemo/Radiotherapy for B-Cell Malignancies." Protocol #92004 302104-A (#22853) Site: Yale New Haven Hospital		
	084		7/16/96		Notification of study site termination for Dr. Richard Champlin at MD Anderson for protocol 92004 302104-A. (22681)		
	085		7/24/96		Add Dr. Michael Lill and Dr. Lee Rosen Protocol entitled "CD34+ Cells from Mobilized Peripheral Stem Cell Collections for Hematologic Rescue of Patients Treated with High-Dose Chemo/Radiotherapy for B-Cell Malignancies." Protocol #92004 302104-A (#22899) Site: UCLA Medical Center		
	086		8/7/96		Submission of IRB approval of protocol change to use Isolex 300i for Dr. Scott Rowley at the Fred Hutchinson for protocol 92004 302104-A (22949)		
	087		8/7/96		Submission of IRB approval of protocol change to use Isolex 300i for Dr. Peter Dreger at Christian-Albrechts Universitat, Kiel, Germany for protocol 92004 302105 (?????)		
	088		.8/15/96		Notification of two manufacturing changes to the Isolex 300i disposable set: 1) sterilization validation method and 2) addition of Mountain Home as an additional manufacturer. (22983)		

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U.S. SUBMISSION INDEX

FDA File # Submission Title	IND/IDE Serial #	Sponsor	Submission Date	Approval Date	Comments		
					Submission Purpose	Referenced MFs, other Submissions	Investigators Baxter Protocol #
	089		09/20/96		Upgrade of software from ver. 1.11 to 1.12 (23184)		
	091		10/1/96		Letter to D. Beitzell as follow up to telephone conference on 9/30/96. (23252)		
	092		10/17/96		Submission of protocol amendments for Dr. Yanovich related to change from Isolex 300sa to 300i. Also submitted the IRB Annual review/approval letter. (23316)		
	093		10/21/96		Submission of institutional protocol and Informed Consent for Dr. Skikne at University of Kansas Medical Center (23339)		
	094		11/4/96		Add Dr. Joseph Uberti Protocol entitled "CD34+ Cells from Mobilized Peripheral Stem Cell Collections for Hematologic Rescue of Patients Treated with High-Dose Chemo/Radiotherapy for B-Cell Malignancies." Protocol #92004 302104-A (#23402) Site: University of Michigan		
	096		11/06/96		Answer FDA's 10/11/96 questions on the May 20, 1996 Ver 1.11 Isolex 300i amendment (23368)		
	098		11/11/96		Add Dr. Robert Preti Protocol entitled, "Peripheral Blood Stem Cells (PBSC) or Isolated CD34+ Cells from Mobilized Peripheral Stem Cell Collections for Hematologic Rescue of Advanced Breast Cancer Patients Treated with High-Dose Chemotherapy." Protocol #92006 302103. (#23442) Site: New York Blood Center		
	099		11/12/96		Response to FDA's request for additional information in support of pending PMA submission. (#23405)		
	100		11/15/96		Amendment to protocol for Dr. Craig Rosenfeld, TOPA, to include patients with T-cell malignancies. Protocol 302104-A (23454)		
	102		11/22/96		Amendment on the manufacturing site, S/W, hazard analysis, etc for the Isolex 300sa instrument. (#23425)		

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U.S. SUBMISSION INDEX

FDA File # Submission Title	IND/IDE Serial #	Sponsor	Submission Date	Approval Date	Comments		
					Submission Purpose	Referenced MFs, other Submissions	Investigators Baxter Protocol #
IDE 5272 (cont.)	103		11/25/96		Submission of IR8 approval for switch from chymo to peptide for Dr. Anthony Ho at UCSD. (23340)		
	104		02/19/97		Letter on CJD. (23874)		
	105		02/18/97		Letter on CJD. (23873)		
	106		3/26/97		Notification of change of manufacturing site (to Mt Home) of disposable set, code FIX1028. (23921)		
	112		5/1/97		Addition of new investigator: Dr. George Somlo at City of Hope to protocol 92004 302103. (24004)		
	113 (FDA 172)		5/1/97		Submission of changes to institutional protocol and subinvestigators at Indiana University, Dr. Rafat Abonour. (24006)		
5272 (cont.)	173		5/21/97		Response to FDA with revised and combined (Dynabeads/CD34) Informed Consent and Investigator letter on CJD issue. (24160)		
	174		06/03/97		Request of teleconference with CBER for the Isolex 300i PMA. (#24151)		
	178 (BB-IDE 7214)		6/30/97		Amendment for +/- protocol (24341) NOTE: FDA has given this submission its own IDE number, BB-IDE 7214.	BB-MF 6283	
IDE 5272 cont.	181		07/21/97		Submission of additional clinical data and product failure for the 300i for determination of submission strategy for the 300i in follow up to 7/10/97 conference call with FDA. (24418)		
IDE 5272 cont.	185		08/07/97		Request for a teleconference and a face-to-face meeting for the Isolex 300i System with FDA. (24494)		
	186		08/20/97		1997 Annual Report (24513)		
	189		10/01/97		Preread Package for for the 10/9/97 face-to-face meeting on the submission pathway and strategy for the Isolex 300i. (24667)		
	192		10/3/97		Notification of cross-referenced DMF BB-MF 5070 amendment (10/3/97). Amendment included change in aseptic filling site (from Hyland to CBL). (#24680)		

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U.S. SUBMISSION INDEX

FDA File # Submission Title	IND/IDE Serial #	Sponsor	Submission Date	Approval Date	Comments		
					Submission Purpose	Referenced MFs, other Submissions	Investigators Baxter Protocol #
	193		10/7/97		Additional information/request for 10/9/97 face-to-face meeting with FDA. (24685)		
	202		01/16/98		Notification of change of ownership from Baxter to BIT (25066)		
	203		01/16/98		Request of extension to reply to the 12/11/97 Dr. Zoon letter for the CJD related information (25075)		
5272 (cont.)	204		02/17/98		Request of extension to reply to the 12/11/97 Dr. Zoon letter for the CJD related information (25167)		
	206		02/28/98		Reply to the 12/11/97 Dr. Zoon letter for the CJD related information (25182)		
	207		03/09/98		Notify FDA of company name change Nexell Therapeutics Inc. (25202)		
	212		04/30/98		Notification of cross-referenced DMF 88-MF 5070 amendment (04/30/98). Amendment included CD34 conformance lot results (CBL Fill) and Appearance specification changes. (#30181)		

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** TOTAL PAGE.17 **

8

Rowley, S. D., Loken, M., Radich, J., Kunkel, L. A., Mills, B. J., Gooley, T., Holmberg, L., Mcsweeney, P., Beach, K., Macleod, B., Appelbaum, F., and Bensinger, W. I. 1998. Isolation of CD34+ cells from blood stem cell components using the Baxter Isolex system. *Bone Marrow Transplant.* 21:1253.

Bensinger, W., Appelbaum, F., Rowley, S., Storb, R., Sanders, J., Lilleby, K., Gooley, T., Demirer, T., Schiffman, K., Weaver, C., Reginald, C., Chauncey, T., Klamet, J., Montgomery, P., Petersdorf, S., Welden, P., Witherspoon, R., Buckner, C. 1998. Factors that influence collection and engraftment of autologous peripheral blood stem cells. *J. Clin. Oncol.* 13: 2547.

Nexell Therapeutics Inc.
Irvine, California 92618 USA

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5/99

Nexell, Isolex, and PR34+ are trademarks of Nexell Therapeutics Inc.
Isolex is registered with the U.S. Patent and Trademark office.
Dynabeads® is a registered trademark of Dynal A.S., Oslo, Norway.

U.S. Pat. Nos. 4,714,680; 4,965,204; 5,035,994; 5,081,030; 5,130,144; 5,443,451; 5,460,493; and 5,536,475.

OIPE JC76 AUG 31 1999 PATENT & TRADEMARK OFFICE

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Code 4R9734 1 Unit

NEXELL

Isolex Magnetic Cell Selection System

DESCRIPTION

Isolex Magnetic Cell Selection System

The Isolex Magnetic Cell Selection System consists of the following components:

- Isolex 300 or 300i Magnetic Cell Selector
- Disposable Set for Isolex 300 or 300i Magnetic Cell Selector
  This product contains dry natural rubber.
- Isolex Stem Cell Reagent Kit for either Selector containing:
  - One vial of Anti-CD34 Monoclonal Antibody
  - One vial of Dynabeads® M-450 Sheep anti-Mouse IgG
  - One vial of PR34+ Stem Cell Releasing Agent

  The packaging of this Reagent Kit contains dry natural rubber.

The Disposable Set and the Reagent Kit are intended for use only with the Isolex Selectors. Refer to the Isolex 300 and Isolex 300i Magnetic Cell Selector Operator's Manuals for a detailed description of the Isolex Selector and the Isolex disposable set.

Isolex Stem Cell Reagent Kit

Isolex Anti-CD34 Monoclonal Antibody
The Isolex antibody is an anti-human CD34 murine IgG, monoclonal antibody with lambda light chains. The antibody is supplied in a 2.5 mL (1.0 mg/mL) sterile, nonpyrogenic phosphate buffered saline solution. **Caution:** This product is manufactured as a colorless solution. Should discoloration occur, product should be returned to Nexell Therapeutics Inc. Slight particulate formation may develop during the shelf life of the product; this has no measurable impact on antibody function.

Dynabeads® M-450 Sheep anti-Mouse IgG
Dynabeads® M-450 Sheep anti-Mouse IgG are paramagnetic, polystyrene beads with affinity purified sheep anti-mouse IgG covalently bound to the surface. The sterile nonpyrogenic suspension contains 10 mL of approximately 4 x 10^8 beads/mL in phosphate buffered saline with 0.1% Albumin (Human), USP.

PR34+ Stem Cell Releasing Agent
The PR34+ Stem Cell Releasing Agent is an octapeptide that is supplied in a 20 mL sterile, nonpyrogenic phosphate buffered saline solution.

PRINCIPLES OF OPERATION

The key steps in the positive cell selection process as described in the Operator's Manual are: *Sensitization, Capture/Rosette, Separation, and Release.*

Sensitization: In the Isolex positive selection procedure, the anti-CD34 monoclonal antibody (the primary antibody) is mixed with cells in suspension to permit binding to CD34+ cells.

Capture/Rosette: Following washing to remove the unbound antibody, Dynabeads® M-450 Sheep anti-Mouse IgG are mixed with the cell suspension. Dynabeads® M-450 have been coated with the sheep anti-mouse IgG (the secondary antibody), which recognizes the murine-derived anti-CD34 primary antibody. This creates bead-target cell rosette complexes.

Separation: A magnetic field is applied to the chamber, enabling the CD34+ cell-bead complexes to be separated magnetically from the rest of the cell suspension.

Release: Following washing in the chamber of the Isolex Disposable Set to remove non-target cells, PR34+ Stem Cell Releasing Agent is introduced to separate antibodies/beads from CD34+ cells. The beads and associated antibodies are retained within the disposable chamber by the magnetic field. The separated CD34+ cells are then washed to remove residual reagents, such as mouse and sheep antibodies, and collected.

INDICATIONS AND USAGE

The Isolex 300 and Isolex 300i Magnetic Cell Selection Systems are indicated for processing autologous peripheral blood progenitor cell (PBPC) products to obtain a CD34+ cell enriched population intended for hematopoietic reconstitution after myeloablative therapy in patients with CD34-negative tumors. Isolex processing reduces the number of non-CD34+ (non-target) cells, including tumor cells, in the autograft compared with unselected PBPC. Clinical studies have not determined whether use of the Isolex 300 or 300i systems will alter progression-free or overall survival.

It is recommended that sufficient peripheral blood be collected to provide at least 2 x 10^6 CD34+ cells per kilogram of patient body weight after CD34+ cell selection. Infusion of fewer cells has been associated with delayed time to platelet engraftment. (See WARNINGS.)

CLINICAL EXPERIENCE

Description of Clinical Studies

The safety and effectiveness of the Isolex System were evaluated in an open-label, randomized clinical study designed to detect clinically significant delays in engraftment with infusion of Isolex-selected CD34+ cells as compared to unselected mobilized peripheral blood progenitor cells (PBPC)[1].

A total of 189 patients with stage II, III, or IV breast cancer who were candidates for high-dose chemotherapy with autologous PBPC rescue were enrolled. Subjects were eligible for randomization if they achieved adequate mobilization (≥ 20 CD34+ cells/μL peripheral blood); 158 patients (84%) met this criterion. A total of 142 were randomized; 76 patients were allocated to receive Isolex-processed PBPC and 66 to receive unselected PBPC. One subject, randomized to Isolex processing, relapsed prior to transplantation; thus engraftment results are reported for 75 patients in the Isolex arm. The protocol also required a minimum of 5 x 10^6 CD34+ cells/kg in the PBPC product in order to undergo Isolex-processing. Sixty-six (87%) of the 76 patients in the Isolex-processed arm had 5 x 10^6 CD34+ cells/kg collected. Ten of the 75 patients (13%) who were transplanted received back-up (unprocessed) PBPC.

Engraftment

The median time to neutrophil engraftment was the same for both arms, but the median time to platelet engraftment was longer for the Isolex arm (Table 1). Also, the Kaplan-Meier curves for time to neutrophil and platelet engraftment were statistically different based on the log-rank test (more rapid time to engraftment in the control

group). Neutrophil engraftment was defined as the first of three consecutive days where ANC > 500/μL; platelet engraftment was defined as the first of three consecutive days where platelets ≥ 20,000/μL without transfusion support.

The 95% confidence interval for the difference between the median time to recovery for unprocessed and Isolex-processed groups indicate that there is a potential 0.0 to 1.0 day delay in median time to neutrophil and a 1.0 to 3.0 days delay in median time to platelet engraftment for patients in the Isolex-processed arm.

Table 1
Engraftment Characteristics

	Unprocessed n = 66	Isolex-Processed n = 75
Median CD34+ Cell Dose	4.9 x 10^6/kg	3.3 x 10^6/kg
Median to ANC ≥ 500/μL* (95% CI) (Range)	10 (9-10) (8-26)	10 (10-11) (9-16)
Median to Platelets ≥ 20,000/μL* (95% CI) (Range)	10 (9-10) (8-62)	12 (11-12) (8-38**)

* Kaplan-Meier estimate
** Not included is one patient who died at day 200 post-transplant without platelet engraftment.

Post-transplant parameters did not show significant differences between the two study arms with regard to days of hospitalization, days of antibiotic therapy and platelet transfusion support required. The difference in the requirement for red blood cell transfusions in the Isolex arm (median of 5.2 RBC units transfused/patient) vs. the unprocessed arm (median of 4.4 RBC units/patient) was significant (p = 0.04).

Platelet engraftment (≥ 20,000/μL) was not documented for one patient who died of progressive disease at day 200 post-transplant.

Limited data are available regarding hematopoietic function at one year post-transplant. All patients in the unprocessed group with one year data (n = 34) had ANC ≥ 1,000/μL, while 2 of 26 patients in the Isolex-processed group had ANC < 1,000/μL at one year. Both patients achieved ANC > 1,500/μL by 15 months post-transplant; one had received unmanipulated PBPC 10 days post-transplant due to infection. All patients in the unprocessed group with one year data had ≥ 50,000 platelets/μL at one year (n = 39), while 4 of 31 patients in the Isolex-processed group did not. None of these patients required platelet transfusions.

Long Term Follow-up

There was no significant difference in the proportion of patients with infection of any severity (56% of unprocessed vs. 67% of Isolex-processed patients) during the first year post-transplant. However, the proportion with moderate or severe infections (33% vs. 53%) in the first year post-transplant was higher in the Isolex arm (p = 0.03). Only one patient was reported with life threatening infections; this was a control patient who received unselected PBPC. Laboratory studies to assess the adequacy of late immune reconstitution were not performed.

The Kaplan-Meier estimates of the median time to progression were 430 days and 398 days in the Isolex and unprocessed arms respectively. The 1-year mortality rates were 18% and 11%, for the Isolex and control arms, respectively. The median survival had not been reached.

DEVICE PERFORMANCE

Composition of Enriched CD34+ Cell Products

Processing of mobilized autologous apheresis products from patients reduced the total number of CD3+ and CD19+ cells by greater than 1,000-fold and 100-fold, respectively, as assessed by immunofluorescence. Device performance parameters obtained from apheresis samples used in the study conducted in patients with breast cancer are summarized in Table 2. The median CD34+ cell recovery (yield) was similar for the two devices, 45% (30%-64%, 25th and 75th percentiles, n=50) and 54% (37%-68%, 25th and 75th percentiles, n=82) for Isolex 300 and Isolex 300i, respectively. The median proportion of CD34+ cells in the processed product (purity) was 90% (80%-96%, 25th and 75th percentiles, n=50, Isolex 300 and 80%-95%, 25th and 75th percentiles, n=82, Isolex 300i) for both device configurations.

Table 2
Device Performance Summary

	Isolex 300 System			Isolex 300i System		
	Pre-selection	Post-selection	Log Depletion	Pre-selection	Post-selection	Log Depletion
TNC x 10^6 Median Range # Samples	261 (39-673) 50	0.3 (0.2-8.5) 50	2.4 (1.2-3.2) 50	226 (57-658) 82	1.4 (0.2-12.5) 82	2.3 (1.4-3.1) 82
CD34+ x 10^6 Median Range # Samples	1.5 (0.3-14.1) 50	0.7 (0.2-9.2) 50	NA	2.4 (0.3-21.1) 82	1.2 (.02-12.1) 82	NA
CD3+ x 10^6 Median Range # Samples	57.5 (4.4-305) 30	0.01 (.001-.01) 30	3.5 (2.2-4.7) 30	53.3 (4.1-152) 54	0.02 (.001-1.8) 54	3.4 (1.7-4.5) 54
CD19+ x 10^6 Median Range # Samples	2.2 (0.1-12.1) 21	0.01 (.001-.04) 21	2.3 (1.8-3.0) 21	1.8 (0.1-129) 20	0.02 (<.001-.7) 20	2.0 (0.2-3.5) 20

Tumor Depletion

Depletion of breast tumor cells has been quantitated using immunocytochemical assays. In eight apheresis products which had been spiked with breast cancer tumor cell lines, tumor cells were reduced > 2,000-fold.

In products from patients with non-Hodgkin's lymphoma and chronic lymphocytic leukemia, tumor depletion was assessed using immunofluorescence assays to identify lymphoma or leukemia cells based on the co-expression of B-cell markers (e.g., CD5/CD19) and/or the exclusive expression of kappa or lambda light chains[2,3,4]. In the twenty procedures with quantitative results, tumor cells were depleted by greater than 200-fold, and in eleven by greater than 1,000-fold.

Tumor cells from patients with multiple myeloma were identified by the high level expression of CD38 using an immunofluorescence assay. In a retrospective analysis of twenty-six quantitative procedures, CD38 bright cells were depleted by 64- to greater than 30,000-fold (mean 4,604-fold). Twenty-one of twenty-six procedures resulted in a greater than 200-fold reduction.

The impact of tumor cell depletion on progression-free and overall survival has not been established in randomized prospective studies.

CONTRAINDICATIONS

The use of the Isolex System is contraindicated in patients whose tumors express the CD34 antigen.

Isolex-processing is not indicated for use with previously cryopreserved and thawed PBPC products. CD34+ cell recovery and viability can be significantly decreased after Isolex-processing with cryopreserved cell products.

WARNINGS

The safety of Isolex-processing in patients with unsuccessful stem cell mobilization (a circulating CD34+ cell number of < 20/µL or with < 5 x 10^6 CD34+ cells/kg in the apheresis products prior to selection, has not been fully studied, thus, is not established.

It is recommended that sufficient apheresis product be harvested to provide ≥ 2 x 10^6 CD34+ cells/kg of patient body weight after selection (see discussion of cell recovery in DEVICE PERFORMANCE). Failure to infuse an adequate number of CD34+ cells can result in delayed engraftment of neutrophils and platelets[5], and potentially engraftment failure.

The recommended CD34+ dose has not been prospectively validated. Further, since CD34+ cell measurements have been shown to vary widely, the value should not be considered to be definitive.

If at any time the user believes that the cells necessary for engraftment remain in the non-target fraction, the non-target fraction may be collected using aseptic techniques and cryopreserved. (see Chapter 4 for the Isolex 300 System and Chapter 6 for the Isolex 300i System for specific details for collecting non-target fractions.)

Handling, processing, or storing cell products under conditions which deviate from the procedures which are specified in the Operator's Manual requires validation to ensure that such modifications will not result in inadequate CD34+ cell yield and/or purity. It is essential that routine training of all users occur at the time that device placement is performed.

Performance failures were reported at a rate of approximately 0.3% between 1995 and 1999. Performance failures may be caused by poor quality apheresis products or not adhering to the instructions for use. Therefore, it is important to follow the instructions for use in the Operator's Manual for the Isolex device and the manufacturer's recommended instructions for use of the collection device (apheresis product).

Excessive cell clumping in the apheresis product has been associated with unsatisfactory device performance (performance failures). Procedures or conditions which promote clumping should be avoided. Although the causality has not been investigated, the following situations have been observed in association with clumping and performance failure: processing of cryopreserved apheresis product (previously frozen and thawed); low cell viability (<90% viability) in the apheresis product prior to processing; elevated platelet count in the apheresis product; and elevated paraprotein level in the apheresis product.

PRECAUTIONS

Potential risks related to additional Isolex processing include the risk of infusion of bacterially contaminated cells that may cause infection (see ADVERSE EVENTS; *Cell Product Sterility*). Use aseptic techniques for all procedures. All selected products should be evaluated for microbial contamination prior to reinfusion.

There is the potential for infusion of foreign proteins which are residual process components, which may evoke an immune response (see ADVERSE EVENTS; *Human Anti-Mouse Antibody (HAMA) / Human Anti-Sheep Antibody (HASA) Response*).

The Isolex Reagent Kit and disposable sets are intended for single use only. Do not reuse components. The fluid pathways of the disposable sets are sterile and nonpyrogenic. Do not use if package integrity is compromised.

Treat all blood products as though they contain an infectious agent. Follow institutional guidelines regarding the handling of infectious agents. Dispose of all materials used in this procedure as biohazardous waste.

ADVERSE EVENTS

Engraftment Delay

Seventeen (22%) of the patients in the Isolex-processed arms had $< 2 \times 10^6$ CD34+ cells/kg in the selected product. Five of these seventeen patients received unselected (back-up) cells to provide a total dose $\geq 2 \times 10^6$ CD34+ cells/kg. The remaining twelve received a total dose $< 2 \times 10^6$ CD34+ cells/kg (selected cells), and their median time to neutrophil engraftment was not significantly different from the median time for the 63 patients in the Isolex arm who received $\geq 2 \times 10^6$ CD34+ cells/kg (11 and 10 days, respectively). However, the median time to platelet engraftment was significantly delayed for patients in the Isolex arm who received $< 2 \times 10^6$ CD34+ cells/kg compared to those who received $\geq 2 \times 10^6$ CD34+ cells/kg (14 and 12 days, respectively). No patients in the unprocessed group received fewer than 2×10^6 CD34+ cells/kg.

Impaired Hematopoietic Reconstitution

There were 5 patients in the Isolex-processed arm with evidence of impaired hematopoietic reconstitution as assessed by blood counts at one year post-transplant. Two of 26 subjects had an ANC < 1,000/µL and 4 of 31 subjects had platelets < 50,000/µL at one year post-transplant; one of these subjects had impairment of both platelet and neutrophil reconstitution. (See CLINICAL STUDIES.) An additional subject died at day 200 post-transplant without evidence of platelet engraftment.

Other Adverse Events

There were limited data collected regarding infusion-related adverse events. There were no reports of serious infusion-related adverse events in patients who received unprocessed PBPC or those who received Isolex-processed PBPC.

Cell Product Sterility

Sterility was assessed for products collected in the randomized study and Nexell sponsored Phase I and II studies[1-4]. Aliquots were cultured for aerobic and anaerobic pathogens. There were 281 Isolex 300-processed PBPC products cultured; of these, 1 product grew gram negative rods on culture. There were 186 Isolex 300i-processed PBPC products cultured of which 2, from different patients, were positive for *Propionebacterium*. There were no reports of clinical infections related to these infusions, although it should be noted that patients were receiving prophylactic antibiotics.

Human Anti-Mouse Antibody (HAMA) / Human Anti-Sheep Antibody (HASA) Response

A theoretical risk is that of infusing residual process components. Residual murine or sheep antibody may evoke an immune response; anaphylactic reactions may occur in patients with hypersensitivity to products of murine or sheep origin.

Serum samples were tested for the presence of human antibodies to murine (HAMA) or sheep (HASA) antibody. Serum samples from patients who received Isolex-processed PBPC were negative for HAMA (n = 15) and were negative for HASA (n = 13) following infusion. There were no reports of anaphylactic reactions in patients who received Isolex-processed products.

INSTRUCTIONS FOR USE

(Refer to the Operator's Manual supplied with the Isolex Magnetic Cell Selectors for Detailed Instructions For Use)

The system components, sample preparation procedure and instrument set-up depend on the specific Isolex System used. The Operator's Manual includes a detailed list of equipment and materials provided and required (see Chapter 4 for the Isolex 300 System; see Chapter 6 for the Isolex 300i System). The Operator's Manual also includes instructions for equipment and materials required, as well as preparation of solutions and samples. It is important to refer to the appropriate Isolex 300 or Isolex 300i Operator's Manual before proceeding.

The starting product for both Isolex Systems is a mobilized autologous peripheral blood progenitor cell product collected by apheresis. When the Isolex 300 System is used, some early processing steps are performed manually prior to selection, and the CD34+ cells are washed and concentrated manually after selection (see Isolex 300 Operator's Manual).

Following selection with either system, an aliquot should be obtained for microbial and other testing, and the CD34+ cell product should be frozen for later use (see Operator's Manual for details).

STORAGE

The Reagent Kit should be stored refrigerated (2 - 8°C) prior to use. Do not freeze.

REFERENCES

1. Chabannon, C., Corrietta, K., Lotz, J.P., Rosenfeld, C., Shlomchik, M., Yanovitch, S., Marolleau, J. P., Sledge, G., Srour, E. F., Burtness, B., Camerio, J., Gravis, G., Lee-Fischer, J., Faucher, C., Chabbert, I., Krause, D., Maraninchi, D., Mills, B., Kunkel, L., Oldham, F., Blaise, D., and Viens, P. 1998. High-dose chemotherapy followed by reinfusion of selected CD34+ peripheral blood cells in patients with poor-prognosis breast cancer: a randomized multicentre study. *Brit. J. of Cancer* 7:913.

2. Dreger, P., Viehmann, K, Von Neuhoff, N, Glaubitz, T., Petzold, O., Glass, B., Uharek, L., Rautenberg, P., Suttorp, M., Mills, B., Mitzky, P., and Schmitz, B. 1999. Autografting of highly purified peripheral blood progenitor cells following myeloablative therapy in patients with lymphoma: A prospective study of the long-term effects on tumor eradication, reconstitution of hematopoiesis, and immune recovery. *Bone Marrow Transplant.* 24: in press.

3. Abonour, R., Scott, K. M., Kunkel, L. A., Robertson, M. J., Hromas R., Graves, V., Lazaridis, E. N., Cripe, L., Sharpure, V., Traycoff, C M., Mills, B., Srour, E. F., and Cornetta, K. 1998. Autologous transplantation of mobilized peripheral blood CD34+ cells selected by immunomagnetic procedures in patients with multiple myeloma. *Bone Marrow Transplant.* 22:957.

Exhibit A

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
1401 Rockville Pike
Rockville MD 20852-1448

OIPE JC79
AUG 31 1999
PATENT & TRADEMARK OFFICE

6 DEC 1993

Our Reference: BB-IDE 5272

Baxter Healthcare Corporation
Attention: Donald A. Baker, Ph.D.
Director, Regulatory Affairs
1720 Flower Avenue
Duarte, CA 91010

Dear Dr. Baker:

We have reviewed your **Investigational Device Exemption** (IDE) for "Stem Cell Selection System (Isolex 300, Baxter), G-CSF (Amgen), Autologous Peripheral Blood Stem Cells (CD34+ Subset), and Chemotherapy," and your study may proceed; however, we have the following comments and requests for additional information:

1. The patients' tumor cells should be screened for the presence of CD34 antigen. Although CD34 has been reported to be expressed by very limited number of tumor cells including ALL and AML, no data have been provided to show that this antigen is absent on Reed-Sternberg cells of Hodgkin's Disease or on B lymphoma cells. Please provide these data.

2. We have noted a low level of mouse and sheep immunoglobulin in the final cellular product. To assess whether this low level is biologically significant, please assess patients receiving PBSC pre and post-treatment for antibodies to mouse immunoglobulins and sheep immunoglobulins.

3. In view of the anaphylactoid responses associated with chymopapain, please describe the prophylactic measures that will be taken prior to administration of the PBSC.

4. The protocol states that additional apheresis procedures will be performed if the target cell collection endpoint is not reached after three daily leukapheresis [Section 6.7.4]. Please state the failure parameters which will disqualify a patient from the study if the minimum collection is not met.

5. Please provide a reference or description of prior studies with the BEAM regimen which was used to estimate mean time of ANC recovery of 18 days for the control group.

6. Please describe the hospital discharge criteria that will be followed at the study site.

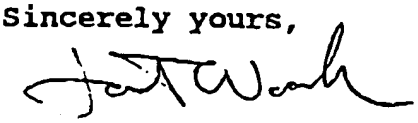
7. Please clarify how you plan to measure days to unmaintained platelet recovery > 20,000.

8. Will a statistical comparison be made between cycle 1 and cycle 2?

9. The proposed clinical trial is not adequate to support licensure of the device for this indication. An adequate trial design would require three arms (G-CSF alone, G-CSF plus peripheral blood stem cell support, and G-CSF plus CD34 positive-enriched peripheral blood stem cells) and would require long-term follow-up to assess the impact on relapse rates, in addition to other safety parameters. Such a trial should also demonstrate the clinical benefit of stem cell support over growth factor support alone and the benefit of CD34 positive-enriched peripheral blood stem cell support over unselected peripheral blood stem cells by clinical parameters.

If you have any questions, please contact Ms. Debra Beitzell at 301-594-3111.

Sincerely yours,



Janet Woodcock, M.D.
Director
Office of Therapeutics Research
and Review
Center for Biologics Evaluation
and Research

Exhibit 1

RECEIVED
MAR 16 1992
MICHAEL J. GRIFFITH, Ph.D.

Office of the Commissioner
Room 14-101 HF-7
5600 Fishers Lane
(301) 443-1306

Food and Drug Administration
Rockville MD 20857

OIPE JC76
AUG 31 1999
PATENT & TRADEMARK OFFICE

March 10, 1992

Michael J. Griffith, Ph.D.
Baxter Healthcare Corporation,
Hyland Division
550 North Brand Boulevard
Glendale, California 91203

Re: Letter of Designation for
Neuroblastoma Bone Marrow Purging System
Our file # RFD-92-1

Dear Dr. Griffith:

By letter dated January 23, 1992, the Food and Drug Administration informed you that the above-referenced request for designation was filed on January 21, 1992, and that the deadline for making the product jurisdiction determination is March 23, 1992.

After conferring with the two affected Centers, I agree with your recommendation and have determined, as provided for under 21 C.F.R. 3.8, that the Center for Biologics Evaluation and Research (CBER) is the agency component designated to have primary jurisdiction for the premarket review and regulation of the Neuroblastoma Bone Marrow Purging System. CBER advises that your product will be regulated under the Medical Device Authorities of the FD&C Act. CBER will consult with the Center for Devices and Radiological Health (CDRH) during the review of this product. You should submit a copy of this letter with your initial application submission to CBER.

Your contact person in CBER for questions on the premarket review and regulation of your proposed product is Dr. Curtis Scribner, Chief, Hematologic Products Branch, Division of Biological Investigational New Drugs, CBER, HFB-230, 7500 Standish Place #250N, Rockville, Maryland 20855, (301) 295-8410. CBER strongly encourages you to request a pre-submission meeting with Dr. Scribner and other CBER staff. This will facilitate your understanding of the nature of the review, including those areas in which CBER will consult with CDRH.

If you have any other questions regarding this matter, please do not hesitate to telephone me at (301) 443-1306.

Sincerely yours,

Amanda B. Pedersen

Amanda B. Pedersen
Product Jurisdiction Officer

Exhibit J

JOURNAL OF HEMATOTHERAPY 7:375–383 (1998)
Mary Ann Liebert, Inc.

OIPE JC76 AUG 31 1999 PATENT & TRADEMARK OFFICE

A Comparison of Two Different Systems for CD34+ Selection of Autologous or Allogeneic PBSC Collections

C.J. STAINER,[1] G. MIFLIN,[1,2] S. ANDERSON,[1] B. DAVY,[1] I.G. McQUAKER,[1] and N.H. RUSSELL[1,2]

ABSTRACT

This study compared CD34 selection procedures using the CellPro CEPRATE® and the Baxter Isolex 300i® systems. Thirty-two procedures were performed, 19 CEPRATE and 13 Isolex. Median starting CD34 percentages were (CEPRATE/Isolex) 0.80% (range 0.24%–7.73%) and 0.85% (range 0.27%–10.17%), respectively ($p = 0.788$). After selection, there was a highly significant difference in purity of the product (CEPRATE/Isolex), 54% and 82% respectively ($p < 0.0001$). There was no significant difference in median recovery (CEPRATE/Isolex), 43% and 50%, respectively ($p = 0.383$). The starting CD34 percentage influenced the purity of the final product, and at high and low starting percentages, the Isolex produced superior purity. Improved efficacy of T cell depletion was observed with the Isolex, a median log depletion of 3.4 compared with 2.9 for the CEPRATE system ($p = 0.012$). In conclusion, the Isolex 300i produced a significantly higher purity CD34+ fraction, even at starting CD34+ levels of <0.5%, with no significant difference in recovery when compared with the CEPRATE system. The associated log T cell depletion is significantly improved with the Isolex system, with possible implications for use in CD34-selected allogeneic transplants.

INTRODUCTION

In the last decade there has been extensive interest, in both the clinical and research fields, in mechanisms for the isolation and characterization of hematopoietic progenitors. The CD34 antigen is a trans-membrane glycoprotein that is expressed on normal hematopoietic cells, 1%–3% of BM mononuclear cells (MC) (1), and 0.1% of normal PBMC (2). Numerous antibodies have been developed against various epitopes of this antigen, for example, 12.8, My10, 9C5, and these have been assigned to the CD34 cluster. Cells within the CD34+ fraction are able to reconstitute normal hematopoiesis following myeloblative chemotherapy (3). Consequently, the CD34 antigen has been the target for use in purification systems to isolate these cells, particularly as it does not appear to be expressed on malignant cells, with the exception of acute leukemias. Vescio et al. (4) found no coexpression on malignant cells in multiple myeloma patients, and Berenson et al. (5) analyzed 36 NHL tumor samples and found no expression of CD34. These diseases are a good target for CD34+ selection, as both BM and PB are frequently contaminated by tumor cells.

Although mobilized PBSC may contain fewer contaminating tumor cells than BM (3,6,7), there are reports that tumor cells are comobilized with progenitor cells (8), and a high percentage of low-grade NHL is reported to have PCR-positive leukapheresis products (9–11). Furthermore, analysis of NHL patients undergoing high-dose chemotherapy found that those receiving successfully purged BM had a low relapse risk after transplant. Craig et al. (12) reported similar findings in patients diagnosed

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[2]Division of Haematology, University of Nottingham, Nottingham, UK.

CD3/CD19 or 5 μl IgG_1 FITC/IgG_1 PE irrelevant antibody in 895 μl of PBS containing 5% BSA. All antibodies were purchased from Becton Dickinson. The cells were washed once with PBS containing 0.01% sodium azide (Sigma). Red cell lysis was performed using 10% FACSlyse (Becton Dickinson), and 1 ml was added to the cell pellet and incubated in the dark for 10 min at ambient temperature. The cells were then washed twice and resuspended to approximately 1×10^6 cells in 0.01% PBS azide.

CD34 analysis was performed using Lysis II software for the FACScan and Cellquest for the FACSCalibur. Using an FL1 versus SSC plot of the CD45-labeled cells, the leukocytes were gated, and 50,000 events were acquired from each tube. The number of CD34+ events was determined using an FL2 versus SSC plot and by drawing a region on the FL2+ population with low side scatter characteristics (24). In more recent cases, the *ISHAGE* CD34 analysis guidelines were used, and this is now our preferred analysis strategy (25). This method avoids overestimation of the CD34 population due to non-specific binding of the HPCA-2 antibody to platelets and debris. However, we have performed a correlation analysis for these two methods and found a strong correlation ($r = 0.98$, $p = 0.0001$), suggesting that they are in agreement for this set of samples and do not bias the results of either system. T cell number was assessed using the same software but a lymphocyte gating strategy. A gate was drawn around the lymphocyte population, and 10,000 events within this region were collected. The T cell number was determined by multiplying the total WBC count $\times 10^8$/kg by the percentage lymphocytes, then the percentage T cells as determined by drawing quadrant regions on the FL1 versus FL2 dotplot. In all analyses, the control percentage was subtracted from the test for the final result reported.

Cellpro CEPRATE CD34 selection procedure

The manufacturer's recommended instructions were followed. Briefly, the initial step was a platelet wash of the pooled or single product, which was divided between two 600-ml transfer packs (Baxter-Fenwal, Deerfield, IL) and diluted in 500 ml of sterile PBS (Gibco, Paisley, Scotland). The bags were centrifuged at 500*g* for 15 min at ambient temperature in a Centra GP8 centrifuge with no brake applied. This was concentrated to a 150-ml cell suspension in PBS containing 0.1% HSA. Biotinylated 12.8 monoclonal anti-CD34 antibody (3 ml) was incubated with the cells for 30 min at ambient temperature. The washing step was repeated to remove unbound antibody. Then cells were resuspended to a final volume of 300 ml in PBS containing 0.1% HSA and connected to the CEPRATE device. The cells are first filtered through a 40 μm Pall filter and pass through the column to the avidin-coated polyacrylamide beads, where the biotin moiety of the CD34-labeled cells is captured. The unbound fraction passes through, and the CD34+ cells (the adsorbed fraction) are released by gentle mechanical agitation of the beads. The enriched progenitors are collected into approximately a 90 ml volume.

Baxter Isolex 300i CD34 selection system

The manufacturer's recommended instructions were followed. Briefly, sterile buffer of PBS containing 0.4% sodium citrate (Baxter) and 1% HSA was prepared. The single or pooled collections were made up to a minimum volume of 250 ml with the buffer. Gammaguard (10 ml) (Baxter) was added and incubated with the cells for 15 min at ambient temperature. During this time, the cells were filtered through a 200-μm blood filter and collected into a 600-ml transfer bag (Baxter). The cells were connected to the sterile disposable set and the automated procedure started with a platelet wash. The platelets were removed when the starting product was transferred to the spinning membrane component of the system. The pore size is such that the MNC were retained and the platelets passed through to be collected into waste bag 1. The rest of the negative fraction was collected into waste bag two. The washed cells were then incubated with the 2.5 mg of 9C5 IgG_1 monoclonal anti-CD34 antibody for 30 min at ambient temperature. Unbound antibody was washed away, and the antibody-coated cells were mixed with sheep antimouse IgG_1-coated magnetic Dynabeads® M450 under slow rotation for 30 min. After magnetic separation of the bead-rosetted CD34+ complex, the beads were released by incubation with the peptide-releasing agent (PR34). The purified product was then washed and concentrated to 100 ml and transferred to a final collection bag.

Statistical analysis

The median values of the parameters analyzed were compared using the Mann-Whitney U-test using SPSS 7.0 for Windows (SPSS Inc., Chicago, IL). All graphs were generated using Microsoft Excel for Windows version 3.1. A p value of <0.05 was considered to be significant.

RESULTS

Recovery and purity of CD34+ cells

A total of 31 patients underwent a total of 32 CD34 selection procedures (Table 1). The CEPRATE system was used for 2 allogeneic and 17 autologous procedures, and the Isolex 300i was used for 2 allogeneic and 11 autologous procedures. One patient with Wegener's gran-

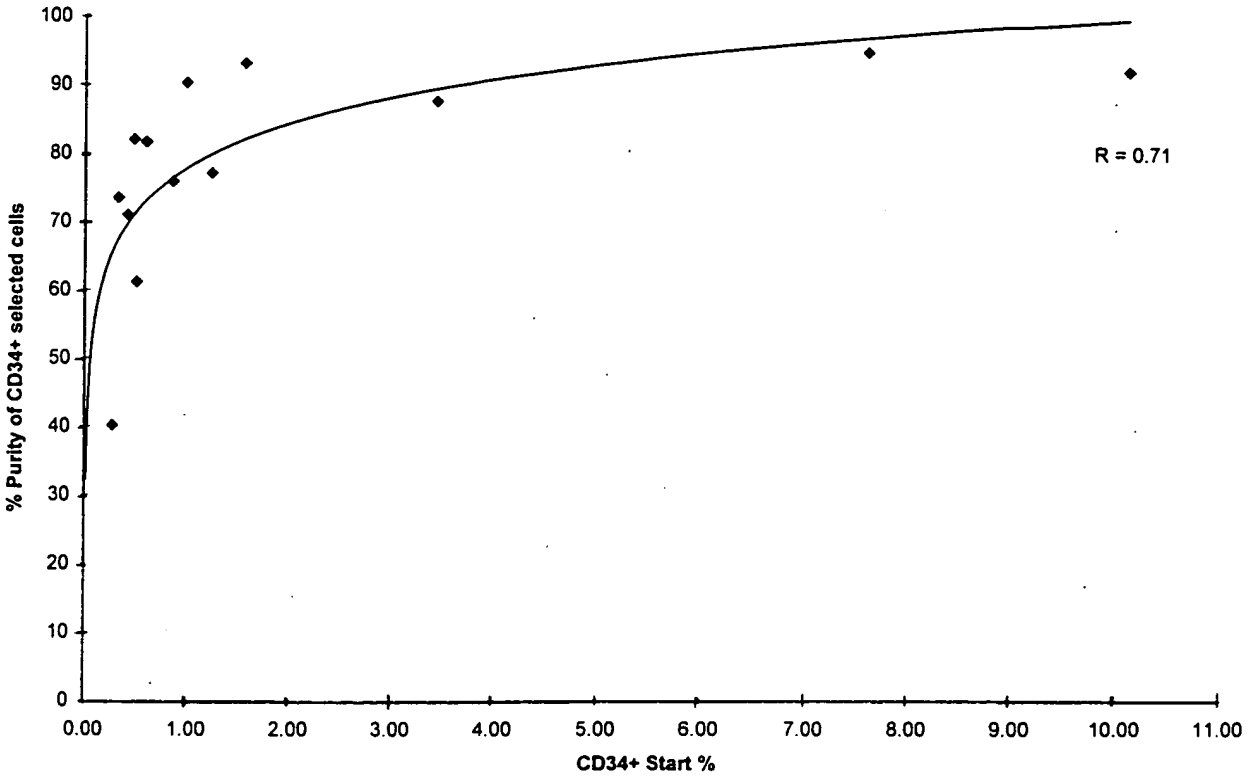

FIG. 2. Comparison of the starting CD34+ percentage with percentage purity of selected cells using the Baxter Isolex 300i system.

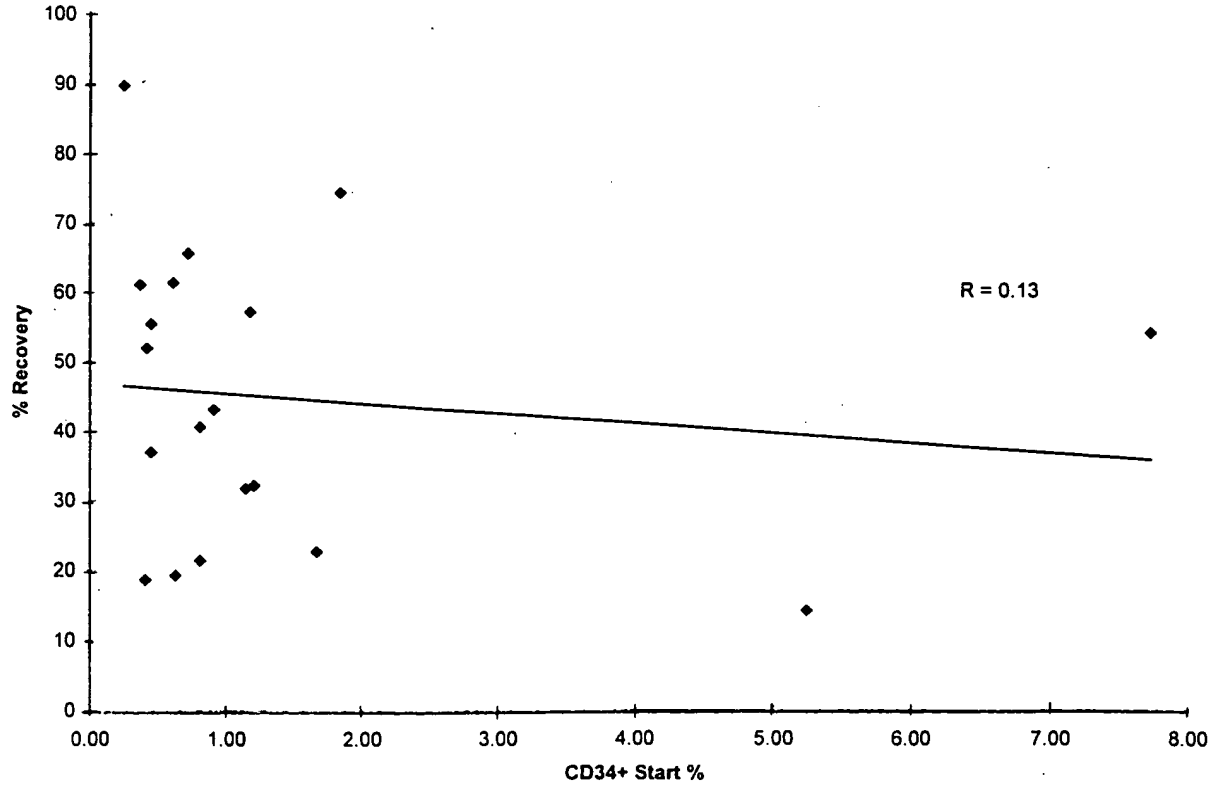

FIG. 3. Comparison of the starting CD34+ percentage with recovery of CD34+ cells using the CellPro CEPRATE system.

DISCUSSION

The purpose of this study was to compare our experience with the semiautomated CellPro CEPRATE selection system with the automated Baxter Isolex 300i selection system. Our aim of infusing $> 1 \times 10^6$/kg CD34+ cells was achieved in 75% of cases. However, in our experience, engraftment has been achieved with lower numbers of CD34+ cells, down to 0.85×10^6/kg, although platelet recovery may be delayed (11). Our major finding was a highly significant difference in median purity of the selected cells achieved with the two systems ($p < 0.0001$). Using the Baxter Isolex 300i, the median purity we achieved was 82%, similar to previously reported results, which are in the range of 83%–98% purity (Table 3). The median purity using the CEPRATE system was 54%, comparable to that previously reported, which has ranged between 42% and 77% (Table 3). The purity of the selected product was significantly correlated with the starting CD34 percentage for both systems (CEPRATE, r = 0.54; Isolex, 300i r = 0.71), which is in agreement with the findings of others (30).

Across the range of starting CD34 percentages, however, the final product contained a higher purity using the Isolex 300i. As a consequence, the Isolex 300i resulted in significantly superior depletion of T cells. Using the CEPRATE system, a median 2.9 log T cell depletion was achieved, compared to a median of 3.4 using the Isolex 300i (p = 0.012). A previously reported T cell depletion of 3.97 logs has been demonstrated by To et al. (31) using the Isolex 300SA, and Clarke et al. (32) reported 2–3 log T cell depletion when using the CEPRATE system. In the small number of CD34+ selection procedures from normal donors that we performed, the T cell number infused was 10^5/kg for the Isolex 300i and 10^6/kg for the CEPRATE system. However, we analyzed T cell depletion in a total of 23 patients, and when the procedures were standardized for the number of CD3+ cells infused into a 70-kg recipient, a similar result was obtained (Isolex, 300i 3.7×10^4/kg, compared with 1.4×10^5/kg for the CEPRATE system). These differences, although small, could have major implications in the results of CD34+ selected allogeneic PBSC transplants, as a T cell dose of $<10^5$/kg is considered necessary to avoid significant GvHD in the setting of fully matched transplants (33), and a lower dose of 4×10^4/kg is necessary for haploidentical transplants (34).

We found no significant difference in the recovery of CD34+ cells, which was 43% and 50% for the CEPRATE and Isolex 300i systems, respectively ($p = 0.383$). Furthermore, we found no improvement in recovery with increasing starting CD34+ percentages when using either system (CEPRATE, r = 0.13; Isolex 300i, r = 0.25), suggesting a limited capacity for these devices. Hohaus et al. have reported an inverse relationship between starting CD34+ percentage and recovery when using the Isolex 300SA (17). These limitations may be partially explained by findings of a study by Farley et al., which optimized bead/target CD34+ cell ratio for use with Isolex 300SA (16). They found that, using a high bead/target cell ratio (500:1), recovery was good (70.1% ± 13.5%), but purity was lower (61.7% ± 12.4%), and the reverse was true at a low bead/target cell ratio (1:1), where recovery dropped to <20% and purity peaked at >70%. Assessment of the recovery of CD34+ stem cells is critically dependent on an accurate determination of the starting CD34+ number. As preselection leukapheresis products often have low CD34+ percentages and an assessment of these rare events has been reported to be associated with a potential high variability of 10% (14), interpretation of recovery results can be misleading unless accurate gating strategies, such as the *ISHAGE* guidelines, are followed, as for this study.

One of the major advantages of the Isolex 300i is that it incorporates an automated cell-washing step both before and after incubation with the antibody. The significance of a good platelet wash before incubation with antibody is unclear. Watts et al. (30) compared a no-wash method with a wash method prior to incubation with the CD34 antibody using the CellPro system and reported no significance difference in CD34+ cell yield and purity. However, all of their purification procedures were performed on the same day as collection. In our study, the majority of procedures required two leukaphereses to be pooled, with the first collection being stored overnight at 4°C. It is possible that formation of platelet aggregates, which may occur with overnight storage, may be significant in compromising the recovery of CD34+ cells, particularly in the allogeneic setting, where the platelet number in leukapheresis products is high. A comparative study is required, but a superior platelet wash before antibody incubation could be significant in improving recovery when using samples for selection that have been stored overnight.

Analysis of Figures 1 and 3, showing CellPro purity and recovery, respectively, in relation to the starting CD34 percentage, indicates that there are two populations clustering above and below the fitted line. These were further studied in an attempt to explain why products of a similar starting CD34 percentage do not consistently achieve an acceptable recovery and purity. We considered assessing whether the initial nucleated cell count and the residual platelets after washing were factors that may limit the capacity of the CellPro device. In the majority of products with low purity (4 of 7) or low recovery (5 of 9), the product had greater than the median initial nucleated cell count. Although a controlled test has not been performed in our laboratory, it may be advantageous to use an extra vial of antibody to improve recovery and purity by increasing antibody/target cell ratio in cases where

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UK

JOURNAL OF HEMATOTHERAPY 8:75–80 (1999)
Mary Ann Liebert, Inc.

A Controlled Comparison of Two Different Clinical Grade Devices for CD34$^+$ Cell Selection of Autologous Blood Stem Cell Grafts

B. BJÖRKSTRAND,[1] B. SUNDMAN-ENGBERG,[1] B. CHRISTENSSON,[2] and G. KUMLIEN[3]

ABSTRACT

Six patients who were to undergo autologous PBSC transplantation with positively selected CD34$^+$ cells were included in this study to compare the efficiency of two devices for clinical grade stem cell selection, the Isolex 300i® (Baxter, Munich, Germany) and CEPRATE® SC (CellPro, Bothell, WA). PBSC were mobilized by chemotherapy and G-CSF and were collected by leukapheresis on a CS3000 cell separator on 2 consecutive days. The two apheresis products were pooled for CD34 selection. The pooled apheresis products from each patient were divided into two equal portions to be separated on each of the two devices. Cell selection was performed according to the manufacturers' instructions. Enumeration of CD34$^+$ cells was performed by flow cytometry using the HPCA-2 MAb. Purity and yield were significantly better with Isolex than with CEPRATE. Median purity was 93.0% (range 80%–98%) for Isolex and 61.5% (range 27%–72%) for CEPRATE ($p = 0.03$); median yields for Isolex and for CEPRATE were 48.0% (range 18%–73%) and 23.0% (range 17%–29%), respectively ($p = 0.03$). The number of CD34+ cells/kg body weight was also significantly higher with Isolex (median 3.8×10^6, range 1.7–5.2) compared with CEPRATE (median 2.35×10^6, range 0.7–4.3) ($p = 0.03$). Thus, the Isolex 300i device gave products of higher purity and recovered a higher proportion of the CD34$^+$ cells in the harvest before separation. The yield was still poor with both devices, however, and further optimization of the technique for clinical grade stem cell selection is warranted.

INTRODUCTION

HIGH DOSE CHEMOTHERAPY RADIOTHERAPY with autologous PBSC transplantation has gained wide applicability in the treatment of malignant hematologic diseases as well as solid tumors. It has been readily demonstrated that both autologous bone marrow and PBSC grafts are frequently contaminated with tumor cells (1–3) and that such contaminating cells can contribute to relapse after autologous transplantation (4). These findings form the theoretical basis for the necessity of purging to eliminate or reduce the number of clonal tumor cells in the autograft. One way to do this is by transplantation of isolated hematopoietic progenitor cells of the CD34$^+$ phenotype. CD34$^+$ cells are selected in vitro by MAb, using different techniques. The most commonly used methods are antibodies bound to magnetic beads (5–7) and adsorption of biotinylated antibodies to avidin particles (8). Clinical grade devices for large-scale cell processing have been developed and used in clinical settings (9,10). These selection devices are to various degrees associated with certain problems, particularly procedure-related cell loss and the CD34$^+$ cell purity of the selected product. The various devices have been compared only on a laboratory scale basis (11).

The purpose of this study was to compare two clinical

Departments of [1]Hematology, [2]Pathology, and [3]Transfusion Medicine, Huddinge University Hospital and Karolinska Institute, Huddinge, Sweden.

grade CD34+ cell selection devices, CEPRATE® SC (CellPro, Bothell, WA), based on avidin-biotin immunoadsorption, and Isolex 300i® (Baxter, Munich, Germany), based on labeling with immunomagnetic beads, in a clinical transplant setting with respect to purity and cell yield in the $CD34^+$-selected PBSC products. Our results show that the products run in the Isolex device had a significantly higher purity of $CD34^+$ cells and a significantly higher yield as compared with the CEPRATE device.

MATERIALS AND METHODS

PBSC were mobilized by chemotherapy and G-CSF in 6 patients, 2 with multiple myeloma, 2 with breast cancer, and 2 with ALL, who were to undergo autologous PBSC transplantation. The cells were collected in blood bags (Baxter Fenwal, Munich, Germany) by leukapheresis using a Baxter CS3000 continuous flow cell separator (Baxter Fenwal) on 2 consecutive days. The apheresis product collected on day 1 was diluted in an equal volume (1:1) of autologous plasma and stored overnight at 4°C. On the second day, the apheresis products from days 1 and 2 were pooled, and one platelet wash was performed as follows. The pooled product was divided into two blood bags, each diluted with PBS to 500 ml. The bags were centrifuged (1800 rpm for 10 min), and the supernatant was discarded. The cells were then diluted in PBS to 300 ml and divided into two equal portions to be separated on each of the two devices. Cells were counted after vital staining with trypan blue. Samples for flow cytometric analysis were collected.

CD34+ cell selection

Cell separation on the two devices was performed according to the manufacturers' instructions, briefly summarized as follows.

CEPRATE SC. The 150 ml cell suspension was diluted with 0.75 ml of 20% HSA (Pharmacia, Uppsala, Sweden), followed by the addition of 3.0 ml of the biotinylated anti-CD34 IgM MAb 12.8. The mixture was incubated for 30 min at ambient temperature, with one gentle mixing after 10 min. After incubation, the cells were washed in 500 ml PBS, centrifugated, and resuspended in 300 ml PBS. The bag containing the cell-MAb mixture was then mounted to the CEPRATE SC device, and the $CD34^+$ MAb-bound cells were automatically selected by being trapped in a column with polyacrylamide beads coated with avidin. After washing, the positive cells were released mechanically with a rotating propeller, and the $CD34^+$ product was collected in a 100 ml bag.

Isolex 300i. The cells were diluted with DPBS (PBS with sodium citrate 12% and HSA 1%) to 300 ml. Human immunoglobulin (Gammagard, Baxter) 500 mg was added, and the cell suspension was filtered through a 200 μm filter (Codan Medizinische Geräte GmbH & Co KG, Lensahn, Germany) before mounting the cell bag to the Isolex 300i device. The following selection procedure was fully automated and run according to the manual. The cells were labeled with 9C5 anti-CD34 IgG MAb for 15 min, followed by the addition of a secondary anti-mouse IgG1 MAb conjugated to sterile paramagnetic microbeads (Dynabeads, Dynal, Oslo, Norway), and incubated for 30 min. The $CD34^+$ cells bound to the MAb-magnetic bead complex were then trapped with a magnet. A competitive peptide was added to the $CD34^+$ cell fraction to release the beads from the cells, and finally the product was collected in a 100 ml bag.

For both devices, samples for flow cytometric analysis and vital cell enumeration were collected from the 100 ml $CD34^+$ cell products. Thereafter, the 100 ml cell fractions from both devices were concentrated to a volume of 4.5 ml by centrifugation, followed by cyropreservation. $CD34^+$ cell enumeration was also performed on the concentrated cell fraction before cryopreservation.

Flow cytometry

Enumeration of CD34+ cells was done essentially according to the recommendations of the Nordic Stem Cell Laboratory Group (12,13).

Sample Preparation. Aliquots of 50 μl (containing up to 1×10^6 cells) from the initial leukapheresis product, the $CD34^+$, and the $CD34^-$ fractions were stained with the HPCA-2 anti-CD34 antibody (clone 8G12) (Becton Dickinson Immunocytometry Systems, San Jose, CA). In separate tubes, cells were stained with PE-conjugated HPCA-2 and FITC-conjugated HPCA-2, respectively. In addition, cells were stained with CD45-FITC and CD14-PE as well as with negative isotype control antibody (Becton Dickinson). After the addition of 20 μl antibody, the samples were incubated for 30 min at ambient temperature in the dark. After staining, erythrocytes were lysed for 10 min at ambient temperature in a fixative-free lysing reagent (8.29 g ammonium chloride, 1 g potassium bicarbonate, and 0.037 g ethylenediaminetetracetate in 1 L deionized water). The cells were then washed twice in PBS + 0.5% BSA + sodium azide before flow cytometric analysis. Samples were kept at ambient temperature and were analyzed within 10–40 min after staining. For the exclusion of dead cells, 5 μl of propidium iodide (PI) (2 μg/ml, Sigma, St Louis, MO) was added to the tube stained with CD34-FITC-conjugated antibody, and the cells were analyzed after 10–20 min.

Flow Cytometric Analysis. Prepared samples were analyzed on a FACScan flow cytometer, using CellQuest software (Becton Dickinson). The threshold was set on

the forward scatter (FSC) signal. The appropriate inclusion of leukocytes (including weakly CD54+ leukocytes) was ascertained by back gating on CD45+ cells. For each sample, at least 50,000 cells were acquired from each tube, aiming at the acquisition of at least 100 CD34+ events. The CD34+ cells were identified by plotting side scatter on the x-axis vs. HPCA-2-PE fluorescence on the y-axis according to the protocol recommended by the Nordic Stem Cell Laboratory Group (12,13). Only cells with distinct CD34 fluorescense and side scatter signals similar to normal lymphocytes were included in the analysis region. In PI-stained samples, the PI-positive cells were gated out, and the fraction of HPCA-2-FITC staining cells was determined. Fluorescent signals in negative controls were excluded by the gating procedure or subtracted from the CD34 count when appropriate.

Statistical analysis

Differences between the CEPRATE SC and Isolex 300i devices with respect to purity and yield of CD34+ cells were analyzed by comparing the numbers and frequencies of CD34+ cells in the positively selected cell fractions from the two devices in each individual patient, using the Wilcoxon signed rank test. Purity is defined as the ratio between the number of CD34+ cells and the total number of nucleated cells in the CD34+-selected cell product. Yield is defined as the ratio between the number of CD34+ cells in the selected product and the number of CD34+ cells in the unselected product.

RESULTS

The results of the selection procedures are summarized in Table 1. Purity and yield, measured on the postcolumn fraction before concentration/centrifugation, were significantly better for Isolex than for CEPRATE. Median purity was 93.0% (range 80%–98%) for Isolex and 61.5% (range 27%–72%) for CEPRATE ($p = 0.03$). Median yields for Isolex and CEPRATE were 48.0% (range 18%–73%) and 23.0% (range 17%–29%), respectively ($p = 0.03$). The number of CD34+ cells/kg body weight was also significantly higher with Isolex (median 3.8×10^6, range 1.7–5.2) compared with CEPRATE (median 2.35×10^6, range 0.7–4.3) ($p = 0.03$). However, the two devices did not differ with respect to the total nucleated cell count in the CD34+-selected fractions. Thus, the higher yield and higher numbers of CD34+ cells achieved with the Isolex device were due to the consistently higher purity of the CD34+-selected products. There seemed to be a relationship between the input percentage of CD34+ cells and the purity of the selected product for both devices, as the 2 patients with the lowest initial percentage of CD34+ cells (MM, 3.0% for both devices; HC, 1.3% for Isolex and 2.3% for CEPRATE) also had the lowest purity (MM, 88% and 39%; HC, 80% and 27% for Isolex and CEPRATE, respectively).

The concentration centrifugation step was associated with a variable cell loss in 5 of the 6 patients. Thus, the yield in the postconcentration sample was median 73% (range 40%–100%) of the yield in the preconcentration sample (data not shown). However, purity was not affected.

DISCUSSION

This small study comparing two devices for clinical scale CD34+ selection shows that the purity and, as a consequence, also the yield of CD34+ cells were significantly superior for the Isolex 300i device compared with the CEPRATE SC device. Purity was consistently very high with the Isolex, exceeding 90% in four of the six separation procedures, whereas the CEPRATE device showed a greater variability. Similar differences in the range of purity for immunomagnetic and immunoadsorption devices have been observed recently in other studies (10,14–16). The overall median purity in the present study (93% and 61% for Isolex and CEPRATE, respectively) also compares with values in other studies using the Isolex (10,14,17,18) and CEPRATE (9,19–21) devices, as well as in an uncontrolled comparative study of these two instruments (22). Because of the small number of patients, it is not meaningful to apply statistics on the relationship between input percentage and postselection purity of CD34+ cells. The preliminary indication of such a relationship in our study is, however, supported by a recently published study including a higher number of patients (22).

Yield was variable and unpredictable with both devices, which also seems to be consistent with findings by other investigators (10,14–16). However, the overall yield for both devices in the present study was lower than seen in some previous studies (9,10,14,16–21). This might be because of patient-related or center-related reasons but does not interfere with the results of the present study, as it was designed as an intrapatient comparison, with each patient being his or her own control.

Flow cytometric analysis is essential for this type of study. The samples from both devices were handled and analyzed in exactly the same way. According to the Poisson distribution, applicable to rare-event analysis, the acquisition of 100 positive events would give a CV(%) of 10%, which is considered acceptable. In various protocols, the recommendation of events to be acquired has varied from 50,000 to 75,000 to 100,000 (13,23–25) nucleated cells. In this study, we required a minimum of 50,000 cells. Furthermore, samples were acquired in duplicate, including FITC-stained CD34+ cells also stained

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for viability by PI, thus giving information of CD34 counts based on replicates. In unpublished comparative studies by S. Siena and B. Brando, the Nordic and the SIHON (24) dual-platform assays with the ProCOUNT (25,26) and STELLer (23) single-platform assays on buffycoat preparations spiked with purified CD34+ cells, ranging in concentration between 0.01% and 2.2%. Similar percentages and absolute CD34+ cell count were obtained in the four assays. Similarly, several other laboratories, including ours, have observed a good agreement between the Nordic and *ISHAGE* assays (unpublished observations).

In conclusion, although purity is good or acceptable, the procedure of clinical grade stem cell selection is still hampered by the high procedure-related cell loss. Furthermore, yield might be deteriorated further by, for example, novel methods using combined positive and negative selection (27). Thus, there is a need for further optimization of the technique for clinical grade cell selection.

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TABLE 1. RESULTS OF PBSC HARVESTS AND CD34+ CELL SELECTION PROCEDURES[a]

Patient body weight (kg)	Total no. CD34+ cells in unselected product, ×10⁶ (%CD34+ cells of TNC)[b]		TNC in CD34+-selected product, ×10⁶		CD34+ cells in selected product, ×10⁶		Purity of CD34+ cells, %		Yield of CD34+ cells, %		CD34+ cells/kg in selected product, ×10⁶	
	Isolex	Ceprate	Isolex	Ceprate	Isolex	Ceprate	Isolex	Ceprate	Isolex	Ceprate	Isolex	Ceprate
MM/62	146 (3.0)	146 (3.0)	122	110	107	43	88	39	73	29	1.7	0.7
KM/52	554 (7.9)	507 (7.9)	280	176	267	126	95	72	48	25	5.1	2.4
HC/70	192 (1.3)	320 (2.3)	167	184	134	49	80	27	70	15	1.9	0.7
CT/78	735 (3.5)	742 (3.5)	214	306	195	188	91	61	26	25	2.5	2.4
LN/55	607 (4.2)	607 (4.2)	300	200	289	125	96	62	48	21	5.2	2.3
FI/89	2240 (30.0)	2240 (30.0)	419	598	411	385	98	64	18	17	4.6	4.3
			p = NS		p = 0.03		p = 0.03		p = 0.03		p = 0.03	

[a]The measurements on the CD34+-selected fractions were performed on samples from the post-column cell fraction, before cell concentration and cryopreservation.
[b]TNC, total nucleated cells.